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HYBRIDIZATION AND GENETICS IN USTILAGO HORDEI AND U. NIGRA [1]

By WAYNE M. BEVER [2]

Associate pathologist, Division of Cereal Crops and Diseases, Bureau of Plant Industry, Soils, and Agricultural Engineering, Agricultural Research Administration, United States Department of Agriculture

INTRODUCTION

It is well known that new strains of fungi pathogenic on plants may result from hybridization of existing species or races. Such strains are known particularly in the rusts and in certain smuts. Although it has been shown that certain species of the genus *Ustilago* on barley (*Hordeum vulgare* L.) may hybridize, it has not been clearly demonstrated that new physiologic races may result. To gain further information on this phase was the purpose of the present investigation.

There are at least three species of the genus *Ustilago* occurring commonly on barley. As early as 1888, investigators (*6*, *20*) [3] recognized two kinds of smut on barley: Covered smut, caused by *Ustilago hordei* (Pers.) Lagerh., and loose smut, caused by *U. nuda* (Jens.) Rostr. In the head smutted with *U. hordei*, the chlamydospores remain enclosed for a time by a membrane; the spore walls are smooth; and the spores normally germinate by producing a promycelium that bears sporidia. In the head smutted with *U. nuda*, the spores are not enclosed long in a membrane but are liberated soon after the heading of the barley. The spores are echinulate and germinate by forming non-sporidia-bearing promycelia. *U. hordei* infects the very young seedlings, whereas *U. nuda* infects the young immature ovary during or soon after anthesis. In 1894, Biedenkopf (*5*) inadequately described what he considered a third species, *U. medians* Bied., in which he noted both types of spore germination described above.

More recently, investigators of barley loose smut in the United States have obtained unexpected results. For example, the long-accepted belief that loose smut of barley is controlled only by the hot-water seed treatment was brought into question when certain workers (*19*, *21*, *35*) obtained partial to complete control of loose smut with the formaldehyde seed treatment. Furthermore, Tisdale et al. (*36*) obtained loose smut infection of barley grown from seed superficially inoculated with what was interpreted to be *U. nuda*, whereas the original work (*10*) with this species had indicated blossom inoculation as the only means of obtaining infection. The possibility that dif-

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[3] Italic numbers in parentheses refer to Literature Cited, p. 57.

ferent physiologic races of *U. nuda* might enter the host by different means was suggested by Tisdale and Tapke (*35*) as an explanation of such results. The correct interpretation of these and similar results obtained by others in seed-treatment experiments was developed by Tapke (*27*) in 1932, when he demonstrated the existence of a second loose smut of barley, which he described and named *U. nigra*. In this and subsequent studies (*29*, *32*, *33*) Tapke reported consistent differences between *U. nuda* and *U. nigra* in method of infection and response to control through seed treatment with surface disinfectants. These two species were shown to differ also in type of chlamydospore germination; the echinulate chlamydospores of *U. nigra* normally germinate by forming a short promycelium bearing four lateral sporidia. Tapke (*33*) has further established the significance of these observations as a basis for the specific designation of *U. nigra*. He has also pointed out that *U. medians* Bied. cannot be accepted as a valid species because it was doubtless erroneously based on a field mixture of *U. nuda* and a sporidia-producing smut that may have been similar to *U. nigra*.

Only a limited amount of work has been done on hybridization between species of barley smut. Ruttle (*24*), on the basis of her own investigations and those of others, divided the barley smut collections she was investigating into seven types, ranging from typical *Ustilago hordei* to typical *U. nuda*. She suggested the hybrid origin of some of the types she observed, but this was necessarily only a speculation as it is obviously impossible to obtain conclusive evidence of hybridization from miscellaneous field collections alone. Allison (*1*), who studied the hybridization of barley smut species, was unable to obtain hybrid spores by inoculating barley with haploid combinations of *U. nuda* and *U. hordei*, but he did succeed in hybridizing *U. hordei* with what he designated as *U. medians*.[4] The smutted heads produced by these hybrids were morphologically intermediate between the covered and loose types but tended toward the loose condition. Moreover, the size of the chlamydospores of these hybrids was intermediate, and the echinulate character of the spores was dominant over the smooth character. While there were some differences in degree of pathogenicity between the hybrids and their parents, these differences were not sufficiently definite to show that new physiologic races had resulted from the cross. Indications, however, were in that direction.

Hybrids between other species of the smut fungi have been extensively studied (*9*, *15*, *16*, *17*, *20*, *38*). In connection with the studies on the barley smuts, it seemed desirable to study in some detail hybrids between *Ustilago hordei* and *U. nigra*. The studies included the following lines of investigation: (1) Spore germination of the parent species and hybrid populations under different environmental conditions; (2) spore measurements; (3) sporidial fusion and nuclear behavior; (4) sexual compatibility; (5) hybridization and segregation for certain characters; and (6) pathogenicity of hybrids and inbred lines and the symptoms caused by them on the host.

[4] The barley smut fungus with dark chocolate brown to black spore mass and with echinulate chlamydospores that germinate by means of promycelium and sporidia (*1*, *p. 15*) has been shown by Tapke (*33*) to be *U. nigra*.

MATERIAL AND METHODS

Ustilago hordei race 6 (*31*) and *U. nigra* races 4 and 6 (*34*) were used in these tests. Chlamydospore material, which had been purified by collecting the spores and reinoculating the same barley varieties over a 3-year period, was supplied by V. F. Tapke. In addition, the parent material was selfed by the writer for five chlamydospore generations before it was used for hybridization studies. The material supplied by Dr. Tapke agreed with the descriptions of the two species. The *U. hordei* material consistently produced the covered type of smutted head on barley; the chlamydospores were smooth and, when germinated on 1.5-percent potato-dextrose agar at room temperature, formed promycelia with sporidia. Likewise consistently, both races of *U. nigra* produced the loose type of smutted head on barley; the chlamydospores were echinulate and, when germinated on 1.5-percent potato-dextrose agar at room temperature (*32*, *33*), formed promycelia with sporidia.

All cultures used were of monosporidial origin. Single sporidia were isolated according to the method described by Hanna (*12*), except that a Chambers micromanipulator was used to hold the isolating needle. Chlamydospores were placed on a 1.5-percent potato-dextrose agar drop on a cover glass. When they had germinated, the sporidia were isolated from the promycelium of individual chlamydospores and cultured separately. Each chlamydospore from which such isolations were made was given a letter and number, and the sporidia were numbered according to their respective positions on the promycelium, beginning at the apex. Thus, a culture labeled H61 originated from *Ustilago hordei* race 6 and from the sporidium taken from the tip of the promycelium. Similarly, N41 originated from *U. nigra* race 4 and from the tip sporidium. The compatibility of the sporidia from individual promycelia was determined by mixing the respective sporidial cultures in pairs in all possible combinations and spreading the mixtures on poured plates of water agar. The presence or absence of fusion of the sporidia was determined by microscopical examination. When fusion occurred, the sporidial lines were considered compatible, and vice versa. Subcultures of the budding sporidia 2 to 3 weeks old were used as the source of inoculum. The cultures were maintained on a medium consisting of 10 gm. of dextrose, 10 gm. of sucrose, 10 gm. of malt extract, the extract from 400 gm. of potatoes, and 18 gm. of agar-agar per liter of water.

Odessa barley (C. I.[5] 934), which is susceptible to the races of smut employed in this experiment, was used in all studies on progeny of segregating hybrids. For studies of range of pathogenicity, the varieties Nepal (C. I. 595), Lion (C. I. 923), and Himalaya (C. I. 1312) were used.

Tapke's (*28*) method, plus a partial vacuum, was used in making both chlamydospore and sporidial inoculations. The seed was first treated in formaldehyde (1 : 320) for 1½ hours, washed in water for 20 minutes, and allowed to dry 2 or 3 days before inoculation. Culture inoculum was prepared by making sporidial suspensions of compatible combinations of monosporidial lines in 10 cc. of distilled water just before inoculation. The sporidial suspension was then poured over the seed to be inoculated, and the mixture was evacuated in a

[5] C. I. refers to accession number of the Division of Cereal Crops and Diseases.

flask for 25 minutes. After evacuation the seed was planted immediately or was allowed to dry and was then stored until planted.

EXPERIMENTAL RESULTS

SPORE GERMINATION

The effects of environmental factors on the germination, on the type of promycelial structure produced, and on the development of primary and secondary sporidia of *Ustilago hordei* have been studied by various workers (*1*, *18*). Similar studies for *U. nigra* have been reported by Tapke (*32*, *33*). Hüttig (*18*) studied the effect of temperature on the chlamydospore germination of a number of species of smut. He found that *U. hordei* would germinate at 0° to 30° C. At 0°, 1°, and 30°, true sporidia failed to develop. No germination was obtained at 35°.

The writer made a study of the effect of various temperatures, from 4° to 36° C., on the percentage of chlamydospore germination in *Ustilago hordei*, *U. nigra*, and two F_2 hybrids between them. The chlamydospore walls of hybrid 1 were smooth, whereas those of hybrid 2 were echinulate. All the chlamydospores were collected and stored under comparable conditions until the time of the test and were then germinated on 1.5-percent potato-dextrose agar in Petri dishes. The results are given in table 1.

TABLE 1.—*Effect of temperature on the percentage germination of chlamydospores of Ustilago hordei, U. nigra, and 2 hybrids between them*[1]

Temperature (°C.)	Age of culture	Germination[2] of chlamydospores of—				
		U. hordei race 6	*U. nigra* race 4	*U. nigra* race 6	Hybrid No. 1	Hybrid No. 2
	Hours	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
4	8	0	0	0	0	0
	36	43	49	0	0	0
	72	83	67	22	5	1
8	8	0	0	0	0	0
	36	44	47	5	1	20
	72		49	41	45	
12	8	20	0	0	0	0
	36	83	21	12	10	30
	72					
16	8	83	0	4	0	1
	36		50			
	72					
20	8	76	13	16	1	3
	36					
	72					
24	8	80	37	16	26	28
	36					
	72					
28	8	85	45	30	30	30
	36					
	72					
32	8	74	5	2	1	0
	36	82	25	27	17	2
	72	82	25	30	17	2
36	8	0	0	0	0	0
	36	50	0	0	0	0
	72	50	0	0	0	0

[1] Blanks in the table indicate that so many secondary sporidia and infection hyphae had developed that it was impossible to make germination counts.
[2] Percentages are averages of 2 replications.

In general, the temperature requirements for germination of the two species and the hybrids were somewhat similar. At 4° C., *Ustilago hordei* started to germinate within 24 hours, whereas *U. nigra* (N4, N6)

germinated more slowly. At this temperature, *U. hordei* produced an abundance of both primary and secondary sporidia, whereas *U. nigra* produced only a small number of primary sporidia and no secondary sporidia. The spores of the hybrids failed to germinate until they had been in culture for 72 hours, and then only a small percentage of the spores germinated and only a few sporidia were produced. At 4°, the percentage germination of the five cultures differed widely.

At 8° C., germination and development of the two species did not differ greatly from those at 4°. The two hybrids started germinating at 36 hours, instead of 72 hours as they did at 4°, but no infection hyphae were observed. The large increase in the percentage germination in the two hybrids at 8°, as compared with 4°, was the outstanding difference between the germinations at these two temperatures.

At 12° C., fusion of sporidia occurred only slightly in *Ustilago hordei* before 48 hours had elapsed and not at all in *U. nigra* or in the two hybrids. In *U. hordei*, the development of infection hyphae started after 48 hours at this temperature; and profuse development of secondary sporidia occurred in both *U. nigra* races and in the hybrids. In *U. hordei*, the percentage germination was not markedly different at 4°, 8°, and 12°. In the *U. nigra* races and also in the two hybrids, percentage germination at 12° and at 8° did not differ very greatly. At 16°, germination occurred within 8 hours in *U. hordei*, *U. nigra* race 6, and hybrid 2. Profuse development of secondary sporidia occurred in all cultures within 24 hours, and at the end of 36 hours sporidial fusion had taken place, with evidence of profuse development of infection hyphae.

At 20° C., a few primary sporidia were showing in *Ustilago hordei* at the end of 8 hours; whereas, in the *U. nigra* races and the hybrids, the promycelium was only one-third to three-fourths developed at that time. At the end of 24 hours, at 20°, the development of secondary sporidia was so profuse that it was impossible to determine the percentage of germination. After 72 hours at 20°, there was profuse development of infection hyphae in all of the cultures. They were most abundant, however, in the cultures of *U. hordei*. In *U. hordei* and *U. nigra*, there was little noticeable difference in growth and development at 20° and 24°. In the hybrids, however, the percentage germination was distinctly higher at 24° than at 20°. In *U. hordei*, at 28°, there was profuse development of secondary sporidia and a few infection hyphae were showing at the end of 8 hours. In the *U. nigra* races and in the hybrids, the promycelia were one-half to fully developed at that time, and a few primary sporidia were produced in *U. nigra* race 6. Profuse development of infection hyphae occurred between 36 and 48 hours. At the end of 72 hours all cultures of both species and hybrids were one solid mat of infection hyphae.

At 32° C., the type of germination was of special interest. The promycelia were short, very knotty, and constricted and failed to produce sporidia. At the end of 48 hours these promycelia had started to disintegrate, and by the end of 72 hours they had completely disintegrated. Promycelial fragments were scattered over the medium as though the promycelia had exploded. No true sporidia were produced, and, with the exception of *Ustilago hordei*, the percentage

of germination was considerably lower. At 36°, spores of *U. hordei* germinated up to 50 percent, while those of *U. nigra* and the hybrids failed to germinate.

SPORE MEASUREMENTS

Measurements were made of 100 chlamydospores and 100 sporidia each of *Ustilago hordei*, *U. nigra*, and the hybrids between them. The results are given in table 2. It is evident that the size, both of chlamydospores and of sporidia of the hybrids, tends to be intermediate between those of the parents (fig. 1).

TABLE 2.—*Measurements of 100 chlamydospores and 100 sporidia each of Ustilago hordei race 6, U. nigra race 4, and F_1 and F_2 of hybrids between them*

Species or hybrid	Mean size of—	
	Chlamydospores	Sporidia
	μ	μ
U. hordei race 6	7.8 × 8.1	5.8 × 9.4
U. nigra race 4	6.8 × 8.2	3.8 × 10.9
F_1 of hybrid No. 1	6.8 × 7.3	
F_2 of hybrid No. 1	7.1 × 7.8	4.2 × 9.5
F_1 of hybrid No. 2	6.6 × 7.2	

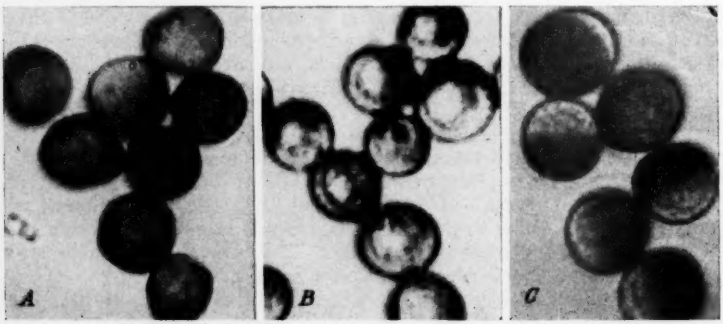

FIGURE 1.—Chlamydospores of (*A*) *Ustilago hordei*, (*B*) F_1 of *U. hordei* × *U. nigra*, and (*C*) *U. nigra*. × 1,800.

SPORIDIAL FUSION AND NUCLEAR BEHAVIOR

The various phases of genetics and cytology of the smut fungi have been studied by numerous workers. Hanna (*13*) and Stakman et al. (*26*) have adequately reviewed the progress of these investigations up to 1929.

Of the early workers, Kniep (*22*) was the first to report that fusion took place only between certain sporidia. He demonstrated the existence of two kinds of sporidia in *Ustilago violacea* (Pers.) Fuckl. and reported that they occurred in about equal numbers. Dickinson (*8*) later confirmed this with *U. levis* (Kell. and Sw.) Magn. and *U. hordei*. Hanna (*13*) and Holton (*15*) obtained infection of oats with crosses between *U. avenae* (Pers.) Jens. and *U. levis*, and found the hybrid spores to be echinulate. Flor (*9*) made crosses between *Tilletia tritici* (Bjerk.) Wint. and *T. levis* Kühn, and reported the hybrid

chlamydospores to be smooth. Holton (*17*), on the other hand, working with the same species, found the hybrid spores to be reticulate.

A number of workers (*1*, *2*, *8*, *11*, *13*, *15*) have described sporidial and hyphal fusion of the various smut fungi. Hüttig (*18*) and Allison (*1*) have described in detail sporidial and other types of fusion and the behavior of the dikaryophytic mycelium of *Ustilago hordei*. However, no detailed study of sporidial fusion in *U. nigra* had been reported previously.

In this study sporidial fusions have been observed between paired sporidia of *Ustilago nigra* and *U. hordei* and between two races of *U. nigra*. Camera lucida drawings of individual sporidia and of fused sporidia are shown in figure 2. The fusion process that was

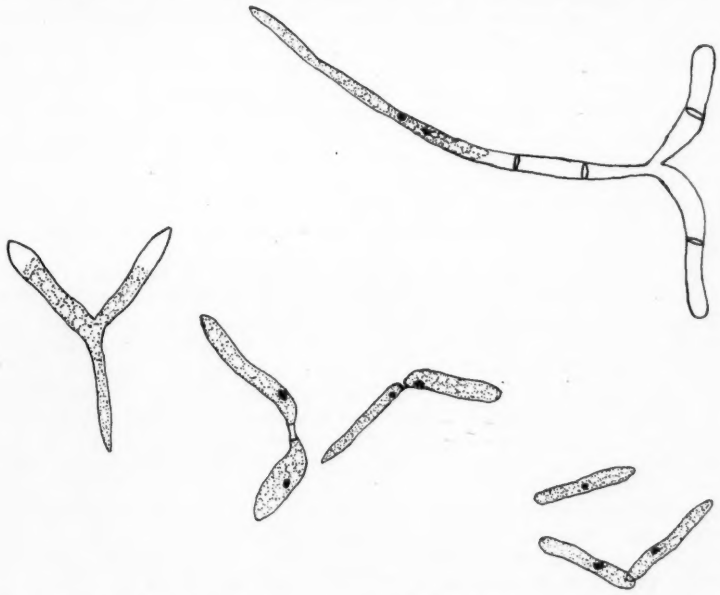

FIGURE 2.—Camera lucida drawings showing stages in the fusion of sporidia of *Ustilago nigra*. × 1,100.

observed was essentially the same as that described by Holton (*15*) for *U. avenae* and *U. levis* and by Rodenhiser (*23*) for *Sphacelotheca sorghi* (Link) Clinton and *S. cruenta* (Kühn) Potter.

In the present study, monosporidial lines of + and − sporidia of *Ustilago nigra* were mixed and spread over poured plates of plain water agar. Observations were made to determine the time required for these sporidia to fuse. It was observed that fusion started within 4 hours and was most active within 4 to 6 hours, depending on the temperature at which the cultures were kept. All the sporidia observed were uninucleate.

The various stages observed in the fusion process between compatible sporidia of *Ustilago nigra* are shown in figure 2. Contrary to observations reported by Holton (*15*) for *U. avenae*, that one sporidium

assumes an active role in the fusion process, all sporidia of *U. nigra* seemed to be equally active; however, it was evident that one sporidium became shorter and broader at the time fusion started. The first development observed was the appearance of a short projection at or near the tip of the sporidium. The projection increased in length until it came in contact with one from a sporidium of the opposite sex, and then fusion followed. Following fusion, the infection hypha was formed (fig. 2).

The fusion of the two sporidia initiates the dikaryophase. Following the completion of the fusion process, infection hyphae were produced. The hyphae in culture are aerial in habit and may not be observed in abundance until after 36 to 48 hours. After 72 hours, the cultures were a solid mass of aerial hyphae, whereas only an occasional aerial hypha appeared in the cultures of paired noncompatible lines. Within a short time after fusion, the protoplasm of the paired sporidia passed into the infection hyphae and migrated toward the tip as the hyphae increased in length. In stained preparations two nuclei that had migrated from the paired sporidia were seen within the cytoplasm. As the protoplasm progressed with the growth of the hyphae, cross walls appeared, cutting off the evacuated basal portions. An indefinite number of these empty cells, usually two or three, immediately behind the protoplasm remained turgid, while those back of that point collapsed and seemed to be without connection with the protoplasm.

The Bauch test, as used by Bauch (*2*, *3*, *4*) for the determination of sex differences between monosporidial lines of *Ustilago violacea*, *U. zeae* (Beckm.) Unger, and *U. scorzonerae* (Alb. and Schw.) Schroet., by Sleumer (*25*) for *U. zeae*, by Tyler (*37*) for *Sphacelotheca sorghi*, and by Rodenhiser (*23*) for *S. sorghi*, *S. cruenta*, and their hybrids, was not found satisfactory by the writer for determining sex differences in *U. nigra*. Even though fusion was profuse, fluffy white mycelium failed to develop, and the compatible combinations could be clearly identified only by the aid of a microscope. Holton (*15*) reported this test to be unreliable for *U. levis* and *U. avenae*.

Sexual compatibility in the various smut fungi has been studied by numerous workers. Christensen and Rodenhiser (*7*) reviewed the progress of these investigations up to 1940. Dickinson (*8*) was one of the first to report the segregation for sex factors to be on a 2 : 2 basis in *Ustilago levis* and *U. hordei*. Hanna and Popp (*14*) and Holton (*15*) found two sex groups in *U. levis* and . *Uavenae*. Allison (*1*) reported two sex groups for *U. medians* [6] and at least two arrangements of the + and − sporidia on a promycelium.

In the present study, experiments were conducted to determine the segregation of factors for sex in monosporidial lines from single promycelia of *Ustilago hordei* and *U. nigra*. Sets of 4 sporidia were isolated from the promycelium of each of 10 chlamydospores of *U. nigra* and from each of 8 chlamydospores of *U. hordei*. Each monosporidial line was cultured separately on the special malt agar described previously. For each species, the 4 monosporidial lines from each chlamydospore were paired with one another in all possible combinations. On the basis of sporidial fusion in culture, 5 of the 6 possible arrangements of the + and − sporidia on the promycelia were observed in *U. nigra* and all 6 arrangements were found in *U. hordei*. The detailed results

[6] See footnote 4, p. 42.

of the matings, given in table 3, show that the segregation of factors for sex in *U. nigra* occurred in the first division of the fusion nucleus in chlamydospores 3 and 4 and in the second division in the others. In *U. hordei*, segregation occurred in the first division in chlamydospores 1 and 2 and in the second division in the others. In both species the segregation for sex factors was in the ratio of 2 : 2, as reported by Allison (*1*) for *U. hordei*.

TABLE 3.—*Sex arrangement of sporidia (4 in each case) taken from promycelia produced by 10 germinating chlamydospores of Ustilago nigra and 8 chlamydospores of U. hordei, determined by pairing in culture all possible combinations of the individual sporidia*

Species from which sporidia were taken and sporidium No.	Segregation of sex factors in chlamydospore No. —									
	1	2	3	4	5	6	7	8	9	10
Ustilago nigra:										
1	−	−	−	+	−	+	−	−	+	−
2	+	+	−	+	+	−	+	+	−	+
3	−	+	+	−	−	+	+	−	+	−
4	+	−	+	−	+	−	−	+	−	+
Ustilago hordei:										
1	−	+	−	−	+	−	+	+	-----	-----
2	−	+	+	+	−	+	−	−	-----	-----
3	+	−	−	−	−	+	+	+	-----	-----
4	+	−	+	+	+	−	−	−	-----	-----

Fusion tests for compatibility grouping were made also between the four sporidial lines from one chlamydospore of *Ustilago hordei* race 6 in all possible combinations with those from one chlamydospore each of *U. nigra* race 4 and *U. nigra* race 6. The results, given in table 4, indicate that there were two compatibility groups between *U. hordei* race 6 and each sporidium of the races of *U. nigra* tested. Undoubtedly other compatibility groups would have been found if sporidial lines from additional chlamydospores from each species and race had been used. It was noted, however, that there was a wide difference in the amount of fusion occurring in the various cultures of the compatible combinations. In some of the cultures there was a small amount of sporidial fusion, while in others fusion was very profuse. The percentage of fusion within a given culture was not determined. However, where there was but little fusion between lines, there also was limited infection in the pathogenicity tests.

HYBRIDIZATION AND SEGREGATION

A number of workers (*9*, *14*, *15*, *16*, *23*, *26*) have reported the production of hybrid chlamydospores as a result of interspecific and intraspecific monosporidial crossing. Allison (*1*) obtained hybrid chlamydospores in a cross between *Ustilago hordei* and *U. medians* [7] and reported that the F_1 produced smutted heads of intermediate type. Holton (*15*) and Hanna and Popp (*14*) obtained hybrid chlamydospores as a result of monosporidial crosses between *U. levis* and *U. avenae*. Flor (*9*) reported hybrid chlamydospores as a result of interspecific monosporidial crosses between *Tilletia tritici* and *T. levis*. Rodenhiser (*23*) secured hybridization between *Sphacelotheca sorghi*

[7] See footmote 4, p. 42.

and *S. cruenta*, and Tyler and Shumway (*38*) between *S. sorghi* and *Sorosporium reilianum* (Kühn) McAlp.

TABLE 4.—*Compatibility (+) and noncompatibility (−) of 4 sporidial lines from a chlamydospore of Ustilago hordei race 6 with 4 sporidial lines from a chlamydospore of U. nigra race 4 and of U. nigra race 6, as determined by fusion*

U. hordei race 6, sporidium No.	*U. nigra*							
	Race 4, sporidium No.				Race 6, sporidium No.			
	1	2	3	4	1	2	3	4
1	+	+	−	−	−	+	+	−
2	−	−	+	+	+	−	−	+
3	+	+	−	−	−	+	+	−
4	−	−	+	+	+	−	−	+

In the present study, crosses were obtained between *Ustilago hordei* and *U. nigra* by inoculating seed of Odessa barley with combinations of monosporidial lines from each species. Chlamydospores from which the monosporidial cultures were obtained were the result of five chlamydospore generations of inbreeding. Inoculations were made also with intraspecific combinations of monosporidial lines from each species. Infection and chlamydospore production were obtained only from combinations that had produced fusion in culture. The intraspecific combinations produced chlamydospores that were characteristic of the species. The F_1 chlamydospores produced by the interspecific cross were echinulate, and the type of smutted heads produced by the *U. hordei* × *U. nigra* crosses ranged from almost typical *U. nigra* to the *U. hordei* type (fig. 3).

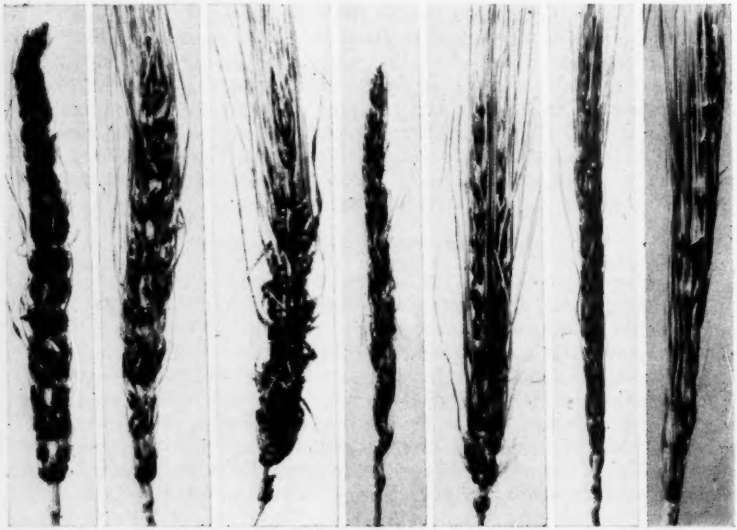

FIGURE 3.—F_1 smutted heads of Odessa barley, caused by inoculating the seed with compatible monosporidial lines from *Ustilago hordei* and *U. nigra*.

Studies were made to determine the manner in which factors for the echinulate-spore character and type of smutted head segregated. F_2 populations were obtained by inoculating Odessa barley with F_1 chlamydospores, and since the sporidia produced by hybrid spores are viable, inoculations also were made with combinations of monosporidial lines from F_1 hybrid spores and with backcrosses to the parent lines. The results of the inoculations with the F_1 chlamydospores are presented in table 5.

TABLE 5.—*Smutted heads of Odessa barley containing F_2 spores, either smooth or echinulate, following inoculation of seed with echinulate F_1 spores of the hybrids Ustilago hordei race 6 × U. nigra race 4 and U. hordei race 6 × U. nigra race 6.*

F_1 inoculum (spores echinulate)	Number of smutted barley heads containing F_2 spores—	
	Smooth	Echinulate
INOCULATED WITH U. HORDEI RACE 6 × U. NIGRA RACE 4		
H61×N41	8	23
H61×N43	19	31
H62×N42	35	35
H62×N44	16	39
H63×N41	0	2
H63×N43	6	10
H64×N42	0	11
H64×N44	40	35
Total	124	186
INOCULATED WITH U. HORDEI RACE 6 × U. NIGRA RACE 6		
H61×N61	7	12
H61×N63	0	11
H62×N62	1	1
H62×N64	0	2
H63×N61	6	10
H63×N63	6	5
H64×N62	10	19
H64×N64	1	2
Total	31	62

It will be noted from the data in table 5 that in the F_2 progeny there was no consistent segregation of smooth and echinulate characters according to the Mendelian 3 : 1 ratio. Only in the hybrid H61×N41 did the segregation approach this ratio. Similar results were obtained when inoculations were made with combinations of compatible monosporidial lines from individual promycelia of F_1 chlamydospores. As shown in table 5, most of the heads produced by the crosses had echinulate spores, but in two cases the number of heads with echinulate and the number with smooth spores were equal, and in two others the number of heads with smooth spores was the greater. Thus, in the majority of the crosses, echinulation was dominant over smoothness, as reported by Allison (*1*).

There is no obvious explanation of these results on the basis of the data at hand, unless the chlamydospores of the *Ustilago nigra* parent were heterozygous. This hardly seems possible, however, as the parent material was inbred for five chlamydospore generations. Moreover, two physiologic races of *U. nigra* were used, and it seems improbable that the chlamydospores of both races were heterozygous for spore markings.

The F_3 chlamydospore generation gave results similar to the F_2, except that in no instance did any cross produce a larger proportion of heads with smooth spores than with echinulate spores.

Two sets of sporidia were isolated from individual promycelia of the echinulate chlamydospores of the F_1 hybrid and used to backcross on each parent.

Pairings were made in all possible combinations and inoculated on Odessa barley. The results secured in the first-generation backcrosses on *Ustilago hordei* and on *U. nigra* are presented in table 6.

TABLE 6.—*Smutted heads of Odessa barley containing first-generation backcross chlamydospores, either smooth or echinulate, following inoculation with the backcrosses Ustilago hordei race 6 × (U. hordei × U. nigra); U. nigra race 4 × (U. hordei × U. nigra); and U. nigra race 6 × (U. hordei × U. nigra)*

Sporidium No. in—		Number of smutted barley heads showing spores—	
Selfed parent	Hybrid parent	Smooth	Echinulate
INOCULATED WITH U. HORDEI RACE 6 × (U. HORDEI × U. NIGRA)			
1	2	51	32
1	4	31	84
2	1	31	54
2	3	4	7
3	2	8	24
3	4	29	68
4	1	12	76
4	3	8	48
Total		174	393
INOCULATED WITH U. NIGRA RACE 4 × (U. HORDEI × U. NIGRA)			
1	1	5	46
1	3	9	28
2	2	4	10
2	4	9	22
3	1	0	16
3	3	0	14
4	2	2	2
4	4	0	4
Total		29	142
INOCULATED WITH U. NIGRA RACE 6 × (U. HORDEI × U. NIGRA)			
1	1	1	3
1	3	4	22
2	2	8	35
2	4	1	6
3	1	2	11
3	3	0	3
4	2	2	1
4	4	1	14
Total		19	95

The results of inoculation with these backcrosses (table 6) show that the segregation of the smooth and echinulate characters of the chlamydospores was not in a simple Mendelian ratio. The smutted-head types produced by the backcrosses on the *Ustilago hordei* parent had a tendency to resemble those produced by *U. hordei* (fig. 4). In some instances the spores were smooth; in others they were echinulate. In

the backcross on the *U. nigra* parent, however, the smutted-head types ranged from the true *U. nigra* type to the true *U. hordei* type, with all degrees of intermediate types (fig. 4). Echinulate spores were not always associated with the *U. nigra* type of smutted head, nor smooth spores with the *U. hordei* type.

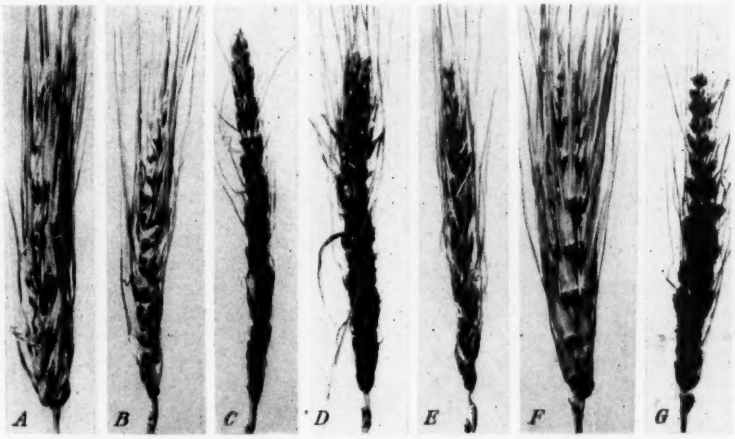

FIGURE 4.—Types of smutted heads of Odessa barley infected by (*A*) *Ustilago hordei* parent, (*B*) first-generation backcross on *U. hordei* parent, (*C* to *F*) first-generation backcross on *U. nigra*, and (*G*) *U. nigra* parent.

PATHOGENICITY

That new pathogenic races of smut fungi may arise through hybridization has been demonstrated by a number of investigators. Holton (*17*) in 1938 reported a new pathogenically distinct race resulting from a cross between *Tilletia tritici* and *T. levis*. Rodenhiser (*23*) also demonstrated that new pathogenic races result from crosses between *Sphacelotheca sorghi* and *S. cruenta*. Allison (*1*) found some evidence that new pathogenically distinct races of barley smut may result from hybridization of *Ustilago hordei* and *U. medians* [8] but they were not strikingly different. Therefore the present studies were undertaken to secure additional information along this line.

Inoculations with combinations of monosporidial lines were made on Odessa barley as previously described. The resulting F_1 chlamydospores, as well as chlamydospores from the parent lines, were used to inoculate separately seed of three varieties of barley, Nepal (C. I. 595), Lion (C. I. 923), and Himalaya (C. I. 1312). These varieties were among those used by Tapke (*29*, *30*) for differentiating physiologic races of *U. hordei* and *U. nigra*. The inoculated seed was planted in the field in duplicate 5-foot rows, and the percentage of smut was determined by head counts. The duplicate rows averaged 200 heads per row. The results are presented as series 1 in table 7.

[8] See footnote 4, p. 42.

TABLE 7.—*Smutted heads of Nepal, Lion, and Himalaya barleys following seed inoculation separately with: (1) Selfed chlamydospores of Ustilago hordei H6, U. nigra N4, and U. nigra N6; (2) echinulate F_1 chlamydospores of U. hordei H6 × U. nigra N6 and U. nigra N4 × U. nigra N6; and (3) echinulate and smooth F_2 chlamydospores of U. hordei H6 × U. nigra N6 and echinulate chlamydospores of U. nigra N4 × U. nigra N6*

SERIES 1 (1939)

Inoculum				Smutted heads on—			Chlamydospore surface in heads of—			Smutted-head type[1] in Nepal, Lion, and Himalaya
Species and hybrid	Smutted-head type[1]	Chlamydospores		Nepal	Lion	Himalaya	Nepal	Lion	Himalaya	
		Generation	Surface							
				Pct.	*Pct.*	*Pct.*				
U. hordei H6	H	Selfed	Smooth	0	21.3	0	Smooth	Smooth	Smooth	H
U. nigra N4	N	do	Echinulate	0	37.0	0	Echinulate	Echinulate	Echinulate	N
U. nigra N6	N	do	do	32.0	9.0	17.0	do	do	do	N
U. hordei H6 × *U. nigra* N6	H, I, N	F_1	do	4.0	3.0	4.0	Smooth or echinulate	Smooth or echinulate	Smooth or echinulate	H, I, N
U. nigra N4 × *U. nigra* N6	N	do	do	10.0	28.0	8.0	Echinulate	Echinulate	Echinulate	N
SERIES 2 (1940)										
U. hordei H6	H	Selfed	Smooth	0	38.0	0	Smooth	Smooth	Smooth	H
U. nigra N4	N	do	Echinulate	0	36.0	0	Echinulate	Echinulate	Echinulate	N
U. nigra N6	N	do	do	37.5	6.0	25.5	do	do	do	N
U. hordei H6 × *U. nigra* N6	H, I, N	F_2	Smooth	8.0	8.0	36.0	Smooth or echinulate	Smooth or echinulate	Smooth or echinulate	H, I, N
Do	H, I, N	do	Echinulate	9.0	7.0	40.0	do	do	Echinulate	H, I, N
U. nigra N4 × *U. nigra* N6	N	do	do	25.0	32.0	15.0	Echinulate	Echinulate	do	N

[1] H = *U. hordei* type; N = *U. nigra* type; I = intermediate.

It is of special interest to note that in the F_1 the interspecific hybrid *Ustilago hordei* H6 × *U. nigra* N6 tended to combine the pathogenic characteristics of its two parents, except virulence. That is, unlike *U. hordei* H6, it was able to attack all three test varieties. The percentages of heads smutted by this hybrid, however, were lower than those produced by either parent. Likewise, the intraspecific hybrid *U. nigra* N4 × *U. nigra* N6, unlike *U. nigra* N4, was able to attack all three test varieties. Thus, both of these hybrids differed from their parents in degree of pathogenicity. Furthermore, some of the heads smutted by the interspecific hybrid contained only smooth chlamydospores while others contained only echinulate ones. These two types of F_2 chlamydospores, and also the F_2 chlamydospores of the hybrid *U. nigra* N4 × *U. nigra* N6, were used separately to inoculate seed of Nepal, Lion, and Himalaya, which was planted in triplicate 5-foot rows. The percentage of smut was based on the total head counts of the triplicate rows. Each 5-foot row averaged 128 heads per row, making a total of 384 heads in a set. The results of the test are presented as series 2 in table 7.

The smut percentages in table 7 indicate that in both series of tests *Ustilago hordei* race 6 and *U. nigra* race 4 showed essentially the same degree of pathogenicity on the three test varieties; that is, neither of these species attacked Nepal or Himalaya and both attacked Lion about equally, whereas *U. nigra* race 6 attacked Nepal and Himalaya rather virulently and Lion only slightly.

In the F_2 of the interspecific hybrid (table 7, series 2), the smooth-spored and echinulate-spored segregates were similar in degree of pathogenicity. That is, both groups produced low percentages of smutted heads in Nepal and Lion and relatively high percentages in Himalaya. They differed, however, in these respects from both of their parents.

Likewise, in the F_2 the intraspecific hybrid *U. nigra* N4 × *U. nigra* N6 differed from its parents in degree of pathogenicity. That is, it produced rather high percentages of smutted heads on all three test varieties; whereas one parent produced a rather high percentage only on Lion and no infection on Nepal and Himalaya, and the other parent produced a low percentage on Lion and rather high percentages on Nepal and Himalaya.

In the F_2, both hybrids tended to combine the pathogenic characteristics of their parents in a manner somewhat similar to that in the F_1, except that the F_2 of the interspecific hybrid produced higher percentages of smutted heads on Himalaya than did either of its parents. Possibly this latter could be considered an example of transgressive segregation; that is, the virulence of the hybrid on Himalaya was greater than that of either parent on this variety.

Numerous microscopic examinations of hybrid chlamydospores showed that most smutted heads of the *Ustilago nigra* type had echinulate spores, whereas those of the *U. hordei* type had smooth or echinulate spores with approximately equal frequency.

DISCUSSION AND CONCLUSIONS

The parent chlamydospore material used in the present investigation was inbred for five chlamydospore generations, after being grown on the same barley varieties for three generations. That the inbred races of *Ustilago hordei* and *U. nigra* used were phenotypically pure is

shown by the fact that they produced smooth and echinulate chlamydospores, respectively, through the several successive generations. Evidence indicates, however, that all the races possessed different genotypes. For example, in the F_2 of the crosses H61×N41 and H61×N43 (table 5), the ratio of smutted heads with smooth chlamydospores to those with echinulate chlamydospores was 8:23 and 19:31, respectively. Similar examples are represented in the crosses H64×N42, H64×N44, H61×N61, and H61×N63. These data suggest that the parental material could have been (1) heterogenous, (2) heterozygous for one or more pairs of factors, or (3) heterogenous and heterozygous for morphological characters. In the F_2 of the crosses H62 and H64 with N42, H62 was more prepotent for the smooth-spore character than was H64. When H62 and H64 were crossed with N44, however, the reverse was true, indicating that factors governing spore markings interacted differently in different combinations.

The F_1 spores were echinulate, ranging from very minute to very distinct echinulation. Although Allison (*1*) reported a 3:1 ratio of echinulate spores to smooth spores in the F_2 from a cross between *Ustilago hordei* and *U. medians*,[9] no evidence of such a ratio was secured in the present studies of F_2 progeny of the cross *U. hordei*×*U. nigra*. The morphology of the heads smutted by the F_1 ranged from the *U. hordei* to the *U. nigra* type rather than being uniformly intermediate in type.

When smooth and echinulate F_2 chlamydospores were used separately as inoculum on Odessa barley, the percentage of smutted heads with smooth spores in the F_3 was practically the same from each lot. This suggests the possibility of some type of an inhibitor active in the F_3. On the other hand, when smooth F_2 chlamydospores of the same cross were used as inoculum on Himalaya, all of the F_3 spores were smooth. This suggests the possibility of a screening out of certain combinations in the F_3, due perhaps to differences in virulence of fused sporidial pairs from the smooth or echinulate spores or to differences in the relative resistance of the two varieties of barley.

It is apparent from the results presented that new physiologic races may result through hybridization of *Ustilago nigra* and *U. hordei*. Fertile hybrids were easily obtained from both interspecific and intraspecific crosses. In the tests on degree of pathogenicity, the hybrids in some cases were more virulent on Himalaya than was either parent. This was true particularly in the F_2 segregates for the smooth and echinulate character of spores. In the cross between the two races of *U. nigra*, there was evidence of a new race from the rather high percentage of infection on all three test varieties inoculated with F_2 chlamydospores.

SUMMARY

The optimum temperature for chlamydospore germination of *Ustilago nigra* and *U. hordei* ranged from 24° to 28° C. Chlamydospores of *U. hordei* germinated over a wider temperature range than did those of *U. nigra*. At 36°, *U. nigra* failed to germinate.

Ustilago hordei hybridized readily with *U. nigra*, as was evidenced by sporidial fusion in culture and by the production of hybrid chlamydospores in the host plants.

[9] See footnote 4, p. 42

Interspecific crosses and backcrosses failed to show simple Mendelian inheritance of chlamydospore markings or pathogenic properties. Nor did the type of smutted head segregate in any such ratio.

The smutted heads produced from a monosporidial interspecific cross ranged in the F_1 from almost typical *Ustilago nigra* to the *U. hordei* type.

The backcrosses of the interspecific hybrids on the *Ustilago hordei* parent tended to produce smutted heads of the *U. hordei* type. In contrast to this, the backcross of the interspecific hybrid on the *U. nigra* parent produced all types of smutted heads, ranging from the true *U. nigra* type to the true *U. hordei* type.

In the F_2 of the interspecific hybrid there was some correlation between the chlamydospore markings and the type of smutted head. Most smutted heads of the *Ustilago nigra* type had echinulate spores, whereas the smutted heads of the *U. hordei* type had smooth or echinulate spores with approximately equal frequency.

Evidence was secured indicating that the range of virulence of *Ustilago nigra* and *U. hordei* may be combined through hybridization, thus forming new physiologic races.

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THE LOCATION AND STATE OF ROTENONE IN THE ROOT OF DERRIS ELLIPTICA[1]

By FRANCIS A. GUNTHER, *associate in the Experiment Station* and FRANKLIN M. TURRELL, *assistant plant physiologist, California Agricultural Experiment Station*[2]

INTRODUCTION

Rotenone and its congeners have come into increasingly widespread usage as the active constituents of a large number of insecticides. A very extensive literature on rotenone and the rotenoids has been amassed; yet very little attention has been given to the histology of tissues containing these compounds in the parent plants (*Derris* spp., especially *D. elliptica* Benth.).[3]

Even though rotenone possesses a water solubility of only 15,000 micrograms per liter at 100° C. (*21*)[4][5], the total water extracts of the roots of plants of *Derris* spp. have, for a long time, been used as fish poisons in the Dutch East Indies and in South America. Van der Laan (*37*), Roark (*30*), Smith, Livengood, and Roberts (*33*), and others have reported that many insects also are very sensitive to the total water extracts from roots of *Derris* spp.

As early as 1919, McIndoo, Sievers, and Abbott (*25*) demonstrated that aqueous preparations containing derris act both as contact insecticides and as stomach poisons. However, a recent trend has been to use insecticidal sprays in which rotenone is dissolved in an oil. Such solvents of rotenone as heavy petroleum oils may cause serious injury to the twigs and leaves of the plant and to its fruits, from physical effects alone,[6] including, for example, the smothering effect of the oil and the adverse effect of the presence of oil in the leaf tissues on the normal physiological functions of those tissues.

While total water extracts of derris roots have been used as fish poisons and insecticides, the relative insolubility of rotenone in water suggested the advisability of reinvestigation of the location and state

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[2] Grateful acknowledgment is made to R. C. Roark and B. Y. Morrison, of the U. S. Department of Agriculture, Washington, D. C., for their aid in securing fresh specimens of *Derris elliptica*, to J. M. Fife, of the U. S. Department of Agriculture Sugar Beet Laboratory, Riverside, Calif., for the loan of an Arntzen micromanipulator; and to V. P. Sokoloff and Wm. Kiebert for literature translations from the Dutch.

[3] The genus *Derris* is a large one comprising many species of tropical woody vines (family Leguminosae, subfamily Papilionaceae, tribe Dalbergieae), particularly abundant in the Old World.

[4] Italic numbers in parentheses refer to Literature Cited, p. 77.

[5] When this investigation was undertaken, the only reference readily available to the authors on the solubility of rotenone in water was that of Jones and Love (*21*). Their value for the solubility of pure rotenone in water was, the writers believed, too low to account for the high toxicity of aqueous extracts of derris roots. It seemed, therefore, that derris resins might exist in the plant in some state permitting greater solubility in water than pure, chemically isolated rotenone.

Since the completion of the present work the data of Frear (*6 p. 80*) on the solubility of pure, chemically isolated rotenone in water at temperatures occurring in nature (i. e., 160 micrograms per liter at 25° C.) have become available. From this work and the work of Hamilton (*14*) it was evident that the solubility of pure rotenone in water was sufficiently high to account for its toxic action on most fish.

The rotenone content of the dry derris root of commerce averages about 5 percent, so that a solubility of 0.016 percent in water would preclude the existence of rotenone in a soluble state in the plant except under very special conditions. This paper shows that at least three special conditions are not satisfied in the plant and that rotenone is found in a resin which exists in the semi-solid state.

[6] Unpublished research, Division of Entomology, University of California Citrus Experiment Station, Riverside. These same physical phenomena seem to play an important role in the killing of the insect pests of plants sprayed with any material containing a heavy petroleum oil. (See also Kraiter (*24*) and Shepard (*31 pp. 204–211*).

of this substance in the plant cell. The present investigation was therefore initiated to obtain information which might form the basis for the development of a much-needed aqueous spray for the control of certain insect pests of citrus.

It was thought that rotenone might be contained in the living plant cell in a partially soluble form. Three possibilities seemed to exist: (a) That the rotenone might be dissolved in an ethereal oil, possibly one of a terpenoid nature; or (b) that it might be dissolved or suspended in the cell sap by means of an unknown solubilizer or mutual solvent; or (c) that it might exist as a glycosidelike compound.

Goodhue and Haller (*10*) found that rotenone-containing plants contained some unidentified sesquiterpenes, a finding which lent plausibility to hypothesis (a). Goodhue and Haller (*9*) and Worsley (*39*) demonstrated the presence of significant amounts of glucosides, similar to saponin, in ground derris root. The fact that the saponins are excellent emulsifying and dispersing agents gave credence to hypothesis (b). With reference to hypothesis (c), rupture of the cell wall in water would release the rotenoid as a glycoside, to form a true solution of the glycoside in water. Plants which synthesize glycosides usually possess cells containing enzymes capable of hydrolyzing these glycosides. Upon maceration of a piece of plant tissue, both glycosides and hydrolyzing enzymes are liberated, with the result that the effective concentration of the former decreases very rapidly. Such a mechanism might explain the failure to isolate glycosidelike rotenoid compounds from air-dried derris roots.

No data in support of hypothesis (b) are available, but the following mechanism of rotenone liberation might be postulated, namely, that the rotenone moiety would be deposited, not by hydrolysis, as in hypothesis (c), but by a change in phase relationships as the solvent in the plant was diluted extraneously with water.

EXPERIMENTS AND RESULTS

LOCATION OF THE ROTENONE-CONTAINING CELLS

Dried roots of *Derris elliptica* were boiled in distilled water for 30 minutes and then sectioned at 120μ under a steam jet on a sliding microtome. Fresh sections were made each day and kept between moist filter paper in a closed dish for use during the one day only, as moist derris [7] root is an excellent medium for mold growth. Unless otherwise stated, this procedure was the one used to prepare all sections from dried root throughout this study. In all such preparations, the water in which the samples were boiled acquired a pronounced milky opalescence; these colloidal extracts were used as the source of part of the material for certain tests to be described later in this paper.

A microscopical examination of sections mounted in water showed the xylem rays, pericycle parenchyma, phloem parenchyma, and xylem parenchyma to be very opaque. This opaque appearance was due to granules (or globules) contained in the cells. When treated with Durham's reagents (*36*, *20*), these opaque, amorphous bodies stained successively red, blue green, brown, and then black. The red coloration was produced by the nitric acid and appeared to

[7] The term "derris," as used here and throughout the remainder of this article, applies specifically to *Derris elliptica;* unless otherwise specified, "derris resin" means the whole rotenone-containing resin (extractives) from *D. elliptica*. This usage refers only to those plants employed by the writers in their investigations.

be permanent if the sections were not treated further, but the addition of ammonia caused the other colors to appear in quick succession.

Sections immersed in 7-percent cupric acetate (Eckerson's test for resins) for 4 days exhibited the characteristic greenish copper-resin precipitate localized in the parenchymatous cells in the xylem rays and xylem, as shown in figure 1, *A*. Precipitates were found, also,

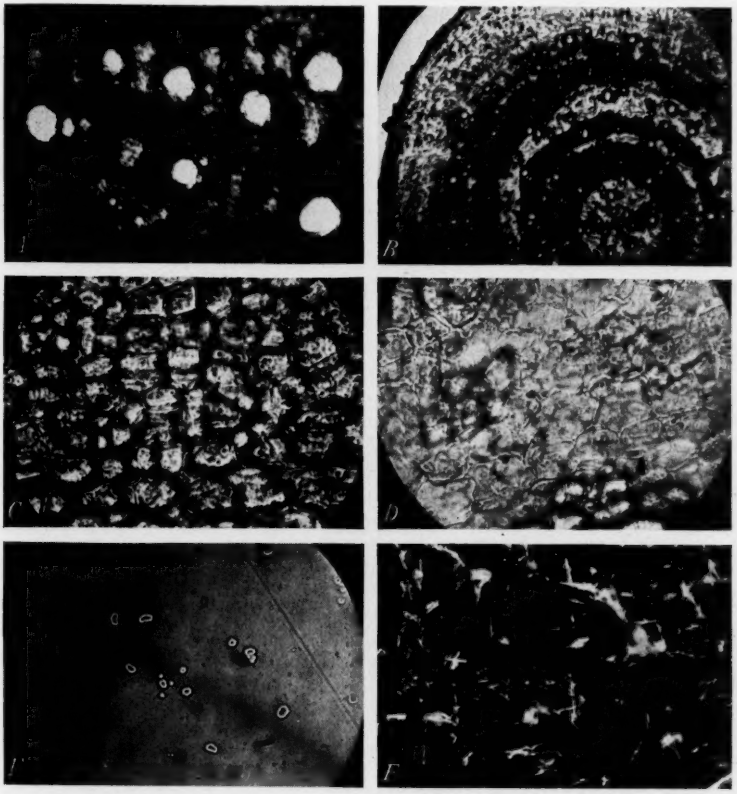

FIGURE 1.—Photomicrographs of sections of root of *Derris elliptica*, following different treatments. *A*, Transverse section of dried root after treatment with cupric acetate to show location of resin deposits. Dark spots in xylem rays and in xylem parenchyma in neighborhood of vessels indicate location of resin which contains rotenone; large, white circular areas are vessels; aggregates of small, light areas are xylem fibers, and grayish areas are xylem parenchyma containing starch. × 46. *B*, Transverse section, 8 mm. in diameter, stained with safranine, showing pentarch structure, the location of starch rings (dark areas), and lignification of parenchymatous cell walls; light rings are regions where rotenone is found. × 10. *C*, Transverse section, showing thick cell-wall structure, small vessels, and starch grains, as found in outer dark safranine-stained ring in *B*. × 217. *D*, Transverse section taken in light portion of dark ring found in *B*, showing thin cell-wall structure and fibers associated with thick-walled cells at edge of photomicrograph; many of the thin-walled cells contain rotenone. × 217. *E*, Starch grains (large) and resin particles (smallest particles in center and at left of photomicrograph) which diffused out of a section of the root into water. × 415. *F*, Mixture of crystalline osazones obtained from the aqueous extract of fresh roots. × 86.

in the phloem and pericycle parenchyma. No precipitate was found in vessels either large or small, or in the fibers.

Resins are of three types, classed as follows: I, common resins; II, oleo resins; and III, gum resins (*17, pp. 864–865*). As rotenone is oil-soluble, it seemed possible that Sudan II, which is an oil stain, might be used in the localizing of the derris resin containing rotenone. In order to characterize the type of staining reaction that might be expected, a number of different resins were tested. The results, presented in table 1 (reagent: Sudan II in 70-percent ethanol, 50-percent acetone solution, saturated), show that the heaviest staining reactions were given by the oleo resins, type II. On the basis of these tests, it is believed that derris resin probably belongs in type II.

TABLE 1.—*Reaction of type resins when treated with reagent Sudan II* [1]

Resin	Type [2]	Reaction
Benzoin	I	Negative.
Guaiacum	I	Few small red spots.
Shellac (liquid)	I	Red.[3]
Canada balsam	II	Do.
Turpentine	II	Do.
Cardolite 627 (phenolic resin)	II	Do.
Olibanum	III	Few small red particles.
Galbanum	III	Numerous red particles.
Derris resin (30-percent rotenone)	Unknown	Red to yellow.
Rosin	do	Small red spots.
Venetian turpentine	do	Negative.

[1] Sudan II in 70-percent ethanol, 50-percent acetone solution, saturated.
[2] Types: I, common resins; II, oleo resins; III, gum resins.
[3] This positive reaction was probably due to the solvent used.

When sections of derris root were treated with Sudan II and washed with 95-percent ethanol, many of the opaque cells stained deep red. These stained parenchymatous cells were found in the xylem, phloem, pericycle, and xylem rays, but vessels and fibers did not show the stain; thus the results of the Durham test [8] were substantiated.

To minimize the possibility of a positive Durham test for rotenone as a result of reaction with a resin rather than with certain constituents of a particular resin, several resins and pure rotenone were tested with concentrated nitric acid followed by 6 N ammonia solution. The results are collated in table 2.

TABLE 2.—*Reactions of type resins in the Durham test*

Resin	Type	Color reaction after treatment with—	
		HNO_3	HNO_3 followed by NH_4OH
Benzoin	I	Red brown	No change.
Guaiacum	I	Deep red brown	Do.
Shellac (liquid)	I	Red brown	Do.
Canada balsam	II	No color	Do.
Cardolite 627	II	Deep red brown	Do.
Turpentine	II	Red brown	Do.
Galbanum	III	Very deep red brown	Do.
Olibanum	III	Red brown	Do.
Derris resin	Unknown	Deep red brown	Blue green.
Rosin	do	Red brown	No change.
Rotenone [1]		Orange red	Intense blue green.
Tannic acid [1]		Deep red brown	Deep brown.
Venetian turpentine	Unknown	Red brown	No change.

[1] Tested for purposes of comparison and elimination.

[8] In the Durham test, substances such as deguelin and malaccol, which are similar to rotenone, give the color test for rotenone. Tephrosin gives no reaction, and toxicarol reacts but weakly.

Derris resin and rotenone alone gave a blue-green color when treatment with nitric acid was followed by treatment with ammonia. Accordingly, it was further substantiated that the Durham test is fairly specific for rotenone and structurally related compounds.

Apparently, the dark, nearly isodiametric parenchymatous cells, which average 70μ in diameter and 3.43×10^5 μ^3 in volume, and which are numerous in the xylem, phloem, and pericycle, and in the xylem rays, contain the rotenone. Under the polarizing microscope, these opaque bodies possess a very slight translucency and have an amorphous appearance; any power of double refraction they may possess is so slight as to be doubtfully discernible. When viewed with transmitted, nonpolarized light, these bodies had an opaque, amorphous appearance, as mentioned previously. When diffused light was employed, that is, when the microscope was placed in front of a window, the cells which contained the opaque bodies appeared sky blue in color, even when untreated with Durham's reagents. For this reason, it was found necessary to use great care in the interpretation of all the Durham tests reported in the present work. These opaque bodies did not stain with safranine, a fact indicating that they were not of an acidic nature.

As a further check on the opaque bodies found in the parenchymatous cells, fresh sections of derris roots were extracted for varying lengths of time (one-half minute to several days) with chloroform, diethyl ether, ethyl alcohol, and acetone. A few minutes in any of these solvents caused the disappearence of most of the opaque bodies, along with a clearing of the section. In each case, the extracted section was tested for the presence of rotenone by means of the Durham test, with only negative results; the corresponding extract was tested by means of the Goodhue (*8*) modification [9] of the Gross and Smith (*11*) color test, always with positive results. This fact indicated that the removal of rotenone from the sections accompanied the disappearance of the opaque bodies.

When the modified Gross and Smith color test was used as a stain in an attempt to stain the rotenone-containing cells directly, diffuse coloring was obtained because of the solvent action of the acetone necessary to the test. The only conclusion that could be drawn was that the color produced was most intense in and around the partially cleared opaque bodies in the parenchyma of the xylem, phloem, pericycle, and xylem rays.

Tapia Freses' (*35*) vanadium-pentoxide-sulfuric-acid test for rotenone was tried, also, as a microchemical test on fresh root sections, but with no success, as the tanninlike substances present in derris were found to give a positive reaction with this reagent. The partial hydrolysis of these tanninlike substances to tannic acid probably produced a purple color reaction with the vanadium reagent, for tannic acid itself reacted positively when tested.

When sections of fresh roots of *Derris elliptica* [10] were used the results of the preceding experiments were confirmed. The sections of fresh roots were prepared like those of the dried roots, except that a minimum amount of water was used to remove the section from the microtome blade. Fresh roots were not boiled or steamed. By

[9] In the present tests it was found that if potassium nitrite is used instead of the sodium nitrite recommended by Goodhue (*8*), the final red color appears to remain unchanged for several days.

[10] These roots were kept packed in moist sphagnum moss while awaiting sectioning.

the use of a microinjection technique,[11] with the aid of a micromanipulator, it was found that the Durham test was positive for the fundamental central parenchyma, xylem rays, phloem parenchyma, pericycle parenchyma, and two concentric parenchymatous rings in the xylem. Vessels, fibers, and certain rings in the xylem, which alternated with the rotenone-containing cells, gave no reaction (fig. 1, *B*).

Since derris resin is quite mobile above 80° C., sections of fresh root were cemented with water glass to a slide maintained at 85° C. by means of an especially built, circulating-water-type slide heater (*13*). The attempt was made to puncture the walls of individual cells and to extract, by suction, the liquid, opaque, resinous, rotenone-containing bodies from the sections with a fine, hollow glass needle operated by a micromanipulator. The extracted rotenone-containing resin, thus isolated, was used for rotenone analysis. Little success was attained, but the opaque bodies in cut cells, when isolated, gave positive results in one test (450 opaque bodies isolated) and negative results in two tests (100 opaque bodies isolated for each of the latter tests). It is believed that the negative tests resulted from the isolation of too small an amount of material to respond to the test.

LOCATION OF THE STARCH-CONTAINING CELLS

When sections were flooded with an iodine-potassium-iodide reagent (0.3 gm. of I_2 and 1.5 gm. of KI, dissolved in distilled water to make 100 ml.), practically 80 percent of the cells stained blue. The presence of starch was thus shown in a large proportion of the parenchymatous cells, except those in the fundamental central parenchyma and in the phloem and pericycle, where only a very limited number of cells showed a positive starch reaction (fig. 1, *C*, *E*). The xylem rays stained dark; a few dark opaque cells were left unstained (fig. 1, *B*). Derris resin and pure rotenone did not react with the iodine reagent.

Other sections were stained with the Sudan II reagent; only a small number (about 10 percent) of scattered cells in the xylem, phloem, pericycle parenchyma, and xylem rays retained the stain (fig. 1, *D*). When the same sections were restained with the iodine reagent, the Sudan II-stained cells did not give a positive starch test, and the continuity of the original staining pattern was retained, an indication that starch and derris resin are not contained in the same cells. A number of large parenchyma cells dispersed in rings in the xylem did not stain with either reagent but remained transparent. These cells may have been cut in sectioning and thus may have lost either starch, rotenone, or contained tannin. Fibers and vessels also remained transparent.

Certain opaque cells in fresh sections became clear and translucent when flooded with acetone. Since acetone does not dissolve starch, the clearing action must have been due to the dissolution of the rotenone-containing resin. These same sections gave positive starch tests only in the uncleared regions, and the cleared areas did not give a test for rotenone when tested with Durham's reagent. These facts confirm Worsley and Nutman's (*41*) view in that they show that

[11] The microinjection needle was connected with an adjustable-height mercury reservoir, so that a very small drop of acid could be ejected onto a very small group of cells in the cross section of the root, the entire operation being viewed under the microscope. Colorations were observed both with and without the addition of ammonium hydroxide.

rotenone is present in cells which do not contain starch. Rotenone does not appear in cells which do contain starch.

Roots up to 6 mm. in diameter are lignified only in the vessel walls and in xylem and phloem fibers. Roots up to 11 mm. in diameter show lignification of the walls of the small and large vessels, the large vessels often being surrounded by many small vessels. Xylem, phloem, and pericycle fibers are also lignified, and the xylem, phloem, and pericycle cells which are parenchymatous but which have thickened walls (fig. 1, *C*) have lignified walls in large roots (above 6 mm. in diameter). Apparently, the cells having the thickened cell walls contain the starch and the thin-walled cells contain the rotenone (figs. 1, *D*, and 2).

The alternation of bands of starch-containing and rotenone-containing cells was noted by Moore and Jones (*26*). They also found that "low carbohydrate" plants, that is, those in the most active growing condition, contained the greatest number of thin-walled cells and the highest percentage of rotenone. These observations seem to correlate well with the presence of thin-walled, rotenone-containing cells and thick-walled, starch-containing cells observed in the writers' root preparations.

Subsequently, it was noted that when sections were treated with dilute nitric acid, as in the Durham test, the milky portions of the section, including the exudate, cleared very considerably. This clearing was similar to that produced by acetone, or by 95-percent ethyl alcohol, and probably was due to its solvent action on the derris resin.

Since nitric acid also probably hydrolyzes the starch in the Durham test, nitric acid, followed by ammonium hydroxide, was added to a water suspension of soluble starch in a macro-test: no color was produced, but the suspension was cleared. This test added further confirmation to the specificity of the Durham test.

LOCATION OF THE TANNIN-CONTAINING CELLS

Three of the known constituents of derris resin, namely, malaccol, sumatrol, and toxicarol, contain free phenolic hydroxyl groups; the other four known constituents, namely, deguelin, elliptone, rotenone, and tephrosin, contain carbonyl groups capable of enolization. The production of a coloration with ferric chloride solution is typical of phenols and enols, but many of them do not give colors.

Since the aqueous extracts both of dried derris root and of rotenone-free dried derris root reacted with aqueous ferric chloride solution (10 percent by weight) to give the green color indicative of the presence of tannins, a series of experiments was carried out in an attempt to locate definite, tannin-bearing cells in root sections. Except for a barely perceptible darkening of the natural color of the solution, neither chemically pure rotenone nor 30-percent derris resin reacted with ferric chloride, either in aqueous suspension or in alcoholic solution. When sections of root were tested with ferric chloride, however, they showed the presence of very numerous darkly stained tannin bodies in the epidermis and a few scattered cells elsewhere. Although a solution of tannic acid itself reacted readily with the reagent, a water extract of the macerated root from which these sections came gave a negative test for tannins. It would thus seem

that the tannins present in the fresh root were not appreciably water-soluble. It could not be determined whether rotenone was present in these tannin-containing cells. The tannin test on root-cork particles was completely negative.

According to Nierenstein (*27, pp. 9–20*) and others, the color reaction between the tannins and iron salts is not specific, since many compounds having phenolic nuclei develop a color with iron chloride. Commercial saponin is distilled water and in the solid state likewise did not react with the ferric chloride reagent, even after 2 hours.

SUGARS AND OTHER REDUCING SUBSTANCES

Samples of the filtered aqueous extract (No. 30 Whatman filter paper in funnel) of fresh sections of root completely reduced boiling Fehling's solution after 20 to 30 minutes. When the filtered extract was made strongly acidic with hydrochloric acid, however, the Fehling's solution was completely reduced in 10 minutes. Another sample of the filtered acidified extract was boiled for 10 minutes, cooled, and made basic with ammonium hydroxide; this mixture completely reduced Fehling's solution in 2 minutes. A third sample of the filtered acidified aqueous extract was boiled for 10 minutes, cooled, and adjusted to pH 7 with dilute sodium hydroxide solution; this mixture, also, completely reduced Fehling's solution in 2 minutes.

The interpretation of these results is that the filtered aqueous extract of derris root, which contained rotenone, contained no uncombined simple reducing sugars but did contain at least one disaccharide, or trisaccharide, or a molecule with a sugar moiety. Acid hydrolysis liberated the reducing sugar. These facts do not eliminate the possibility that rotenone could exist as a glycosidelike compound, as indicated in hypothesis (c) (p. 62), although almost any plant examined will behave in this manner.

In order to characterize the sugars produced by acid or alkaline hydrolysis, the following tests were carried out. To 4-gm. samples of the (Seitz) filtered aqueous extract of ground derris root, each containing approximately 0.2 gm. of solids in solution, was added 0.4 gm. of phenylhydrazine hydrochloride, 0.6 gm. of crystalline sodium acetate, and 4 ml. of distilled water. The osazones were then prepared and precipitated in the manner of Shriner and Fuson (*32, pp. 37–38*) and of Huntress and Mulliken (*18*). The time required for the first precipitation of osazone material varied between 20 and 30 minutes. After filtration and drying at room temperature, the mixture of crystalline osazones resulting from this test (fig. 1, *F*) was compared by the time required for precipitation and microscopically with the pure osazones of arabinose, fructose, galactose, glucose, maltose, saccharose (sucrose), and xylose. Glucosazone was the only derivative definitely identified, although a few scattered crystals which may have been xylosazone were discernible. The paucity of these crystals precluded a positive identification, particularly since the osazone characterization for the "analysis" of a mixture of sugars is not too reliable, owing to the possible formation of mixed crystals, or the deformation of the normal habit of the individual crystals as a result of the presence of foreign substances in solution. With this one possible exception, however, the osazone precipitate presented the uniform crystalline appearance of glucosazone.

Glucose, fructose, mannose, cellobiose, maltose, and sucrose yield identical osazones. The three disaccharides mentioned (cellobiose, maltose, and sucrose) hydrolyze in acid solution to yield either glucose alone or glucose plus fructose. Since the aqueous extract of derris root was shown above to contain no simple reducing sugars, and since maltose and cellobiose are reducing disaccharides (reducing Fehling's solution readily), it may be that the sugar probably present as such in the extract was the disaccharide sucrose, which was hydrolyzed, through the acid treatment in the preparation of the osazones, into glucose and fructose.

Since rotenone and its congeners were removed from the aqueous extract with the Seitz filter and were not detected in the sugar solution, it was concluded that they did not exist as glycosidelike compounds in the plant, as suggested in hypothesis (c) (p. 62).

SAPONINS

A mixture of powdered or shredded derris root and water forms a remarkable amount of moderately stable foam when shaken. After Seitz filtration, the clear yellow to yellow-brown filtrate retains this ability to foam. In addition, derris dust causes sneezing, irritates the throat and nasal passages, and makes the eyes smart unpleasantly. These properties, in general, are characteristic of the saponins. In order to prove the presence of saponins or saponinlike substances, 100 gm. of powdered derris root was shaken with 500 ml. of distilled water, macerated for 1 hour, and then filtered through a Seitz filter (pad No. 6). This clear yellow filtrate, which contained no suspended matter, as indicated by the absence of the Tyndall effect, was used in all the following saponin tests.

To 9 ml. of this aqueous solution, which possessed a pH of 5.5 to 6.0 (nitrazine paper), was added 1 ml. of a light-medium grade petroleum spray oil; upon vigorous agitation, this mixture formed a very stable emulsion. A similar stable emulsion resulted when kerosene was substituted for the previously mentioned spray oil.

Although this filtrate did not precipitate when tested with 25 percent, or with saturated, ammonium sulfate solution it did yield a voluminous yellowish-brown precipitate with both saturated neutral lead acetate solution and saturated basic lead acetate solution. It reduced ammoniacal silver nitrate solution slowly. When 5 ml. of this aqueous extract was added to 10 ml. of a solution made by dissolving 5 gm. of potassium ferricyanide and 0.5 gm. of ferric chloride in 1 liter of distilled water, the resulting mixture turned greenish blue immediately, and then slowly deposited insoluble ferric ferrocyanide (Turnbull's blue) over a period of 2 hours. Another 5 ml. of the extract was evaporated to dryness, and the tan residue was divided into two portions. To one portion was added 2 drops of concentrated sulfuric acid, with the development of a reddish-purple color; to the other was added 2 drops of a solution composed of equal parts of concentrated sulfuric acid and ethyl alcohol containing a trace of ferric chloride, with the development of a red-brown color and a slight fluorescence.

To another 9 ml. of the aqueous extract was added 0.9 gm. of sodium chloride. After solution had occurred, 3 drops of fresh defibrinated beef blood was added. No hemolysis was apparent

after 2 hours, but some dissolution of the erythrocytes had occurred after 12 hours at room temperature. Chopra and Roy (3) found, however, that dilute (0.05-percent, or less) solutions of certain saponins and other hemolysins were completely inactivated by Seitz filtration. Another mixture of derris dust and distilled water was accordingly prepared and centrifuged until clear. The hemolysis test was repeated on a 9-ml. portion of this clear, supernatant liquid, with negative results, but again some hemolysis was apparent after 12 hours.

These experiments, in toto, indicate that there are one or more saponins present in derris roots. Ammonium sulfate does not precipitate these saponins from their aqueous solution, but they exhibit all the other characteristic reactions of their group. These particular saponins are slowly hemolytic. All the above experiments were checked as to type by means of parallel tests with a 0.1-percent aqueous solution of commercial saponin, with results as shown in table 3. Since saponins are definitely proved to be present in the root of *Derris elliptica*, it seems possible that these substances may exert a solubilizing action upon rotenone, and that hypothesis (b) (p. 62) may be given some credence.

TABLE 3.—*Comparison of saponin reactions of derris preparations and of commercial saponin preparations*

Test	Derris preparation	Saponin preparation
Foaming property	Positive	Positive.
Physiological effects [1]	do	Do.
Acidity of aqueous solution (nitrazine)	5.5–6.0	6.5–7.0.
Oil-emulsifying ability	Positive	Positive.
Precipitation by ammonium sulfate solution	Negative	Negative.
Precipitation by neutral lead acetate solution	Positive	Positive.
Precipitation by basic lead acetate solution	do	Do.
Reduction of ammoniacal silver nitrate solution.	Weakly positive	Weakly positive.
Reduction of ferricyanide solution	Slowly positive	Positive.
Reaction with concentrated sulfuric acid	Purple-red color	Red color.
Reaction with concentrated sulfuric acid plus ferric chloride.	Red brown (fluorescence)	Red brown (fluorescence).
Hemolytic power	Slowly positive	Positive.

[1] Causing throat irritation, sneezing, etc.

THE STATE OF DERRIS RESIN IN THE PLANT

When dried fragments of derris root were steeped in cold water for any length of time, it was noticed that the slightly acidic aqueous phase quickly acquired a milky appearance and exhibited the Tyndall effect; when fresh derris roots were cut or bruised, a milky exudate appeared immediately; when fresh derris fragments were placed in water, the aqueous phase acquired a milky appearance within a few minutes and exhibited a pronounced Tyndall effect. Under the low power of the microscope, both the exudate and the aqueous "milk" showed the presence of particles more or less equal in size, all of which seemed to polarize light under crossed Nicols. Under high power (×744), two sizes of particles were distinguishable: The larger ones (granules) were from 7μ to 38μ in diameter, and the smaller ones (globules) were from 0.8μ to 3.9μ in diameter, measured under the oil-immersion objective (×910). The larger particles were very much in the minority and were approximately 10 times the

size of the smaller ones. Only these large particles exhibited birefringence under high power. When this suspension was treated with iodine-potassium-iodide solution, the large particles stained dark blue and lost their birefringence. The addition of 6 N nitric acid to the "milk" slowly cleared it of all particles, as could be readily observed under the microscope by running small amounts of the acid under the cover glass with a pipette. Similar experiments with acetone dissolved the small particles and left the large particles. These tests indicate that the large particles are starch grains and that the small particles are resinous in nature. A photomicrograph of the particles is shown in figure 1, *E*. The small particles exhibited Brownian movement; the large ones did not.

Several samples of both dried and fresh derris root were thoroughly triturated and macerated, separately, with distilled water. After steeping for several days at room temperature, each sample was filtered, first through an ordinary qualitative filter paper to remove fragments of the secondary plant structure, and then through a Seitz filter (pad No. 6) with 25 pounds pressure and 4 cm. vacuum. The clear filtrates were straw yellow in color, were free from colloidal material, and did not exhibit the Tyndall effect. A portion of each filtrate was evaporated to dryness on the steam bath; the residues were tested for the presence of rotenone, both by the Durham test and by the modified Gross and Smith test, with completely negative results in each case. Other portions of unevaporated filtrate were combined (about 20 ml.) and extracted twice with 20-ml. portions of carbon tetrachloride. The combined carbon tetrachloride extracts were evaporated to dryness on the steam bath, 20 ml. of 95-percent ethyl alcohol was added, and the solution was again evaporated to dryness. Another 20 ml. of ethyl alcohol was added to the residue, and the evaporation was repeated. When dissolved in acetone and tested with the modified Gross and Smith reagents, the residue showed complete absence of rotenone and deguelin. Other portions of the filtrate were acidified with hydrochloric acid, evaporated to dryness, and tested for rotenone, with negative results.

The filter pad from each filtration showed a yellow-brown deposit matted in the fibers of the pad. This deposit gave a very strong starch test with iodine-potassium-iodine solution. The deposits from four pads were extracted for 30 minutes with acetone; the acetone extract showed a very high concentration of rotenone when tested by the modified Gross and Smith test, or by the Durham test after removal of the solvent. Another pad with residue, tested directly with Durham's reagents, showed a high rotenone content.

In another experiment, 10 gm. of fresh derris root was shredded into a Waring blendor and extracted with 400 ml. of distilled water for 3 minutes at 12,000 revolutions per minute . The resulting suspension was filtered through qualitative paper, and the filtrate was refiltered through the Seitz filter (pad No. 6). After this filter pad had been dried at room temperature, it was extracted at room temperature with 50 ml. of dry diethyl ether in the dark for 80 days. The ether was removed from the resulting clear extract, in vacuo, and a tan-colored semicrystalline residue, weighing 0.25 gm. (2.5 percent by weight), was left. This residue possessed the odor characteristic of derris resin; it softened at 50° C. and was very motile at 100°. Equal

quantities of this material and of commercial 30-percent derris resin were mixed and warmed slowly alongside a comparison tube of the residue; the contents of both tubes showed similar physical properties (viscosity and appearance) through a range of 0°–100°.

These results indicate that rotenone exists in conjunction with the other components of derris resin within the plant cells as discrete globules of rotenone-containing resin.

THE CHARACTERIZATION OF DERRIS RESIN AS AN OLEO RESIN

As was mentioned earlier, tests with an oil stain indicated that derris resin is an oleo resin. Accordingly, it should be possible to isolate an "oil" from derris resin.

Steam distillation of 300 gm. of a commercial derris resin (30-percent rotenone) from *Derris elliptica* yielded 12.5 gm. (2.8 percent by weight) of a clear, faintly yellow oil with a musty odor. Atmospheric distillation yielded about 11 gm. of cis-dichloroethylene and a small amount of an intense-yellow liquid residue. The dichloroethylene appeared by virtue of the fact that it is one of the solvents used commercially to extract the "resin" from rotenone-bearing roots; it was characterized here by its physical and chemical properties. Vacuum distillation of this liquid residue yielded a very small amount of a clear, colorless, evil-smelling liquid which boiled at 47°–50° C./27 mm. This viscous liquid contained no halogen and burned with a very smoky flame; it slowly reduced Schiff's reagent.

Steam distillation of 100 gm. of finely ground dried roots of *Derris elliptica* (6-percent rotenone) yielded traces of a colorless oil and a white waxy substance. The total distillate was extracted with ether, and this extract was dried over anhydrous magnesium sulfate. Approximately 0.1 gm. of an orange-yellow, oily material remained after removal of the solvent. This residue, which possessed the same evil odor as the residue of oil from the commercial derris resin mentioned above, contained no halogen, burned readily with a smoky flame, and slowly reduced Schiff's reagent.

The properties of these apparently identical oils from *Derris elliptica* suggest that they contain a terpenoid substance which possesses one or more carbonyl groups.

DISCUSSION AND CONCLUSIONS

Koolhaas (*23*), by determining chemically the rotenone content of bark and wood parts of derris root, found that there was generally a slightly larger rotenone content in the wood than in the bark, although the bark also contained a considerable amount of rotenone.

Georgi and Teik (*7*) confirmed these findings, both by determining the total ether extractives of bark and wood separately and by determining chemically the amount of rotenone in bark and wood; these determinations were based on dry weight. In addition, they found the proportion of dry weight of the bark and of the wood to the whole root. Their figures indicated, also, a little less rotenone or ether extractives in the bark than in the wood.

Several attempts have been made to find a distinct and characteristic microchemical test to distinguish a root rich in rotenone from one poor in rotenone. Van der Laan (*37*) found that a very sensitive color reaction[12] for solutions of rotenone, described by Jones and Smith (*22*),

[12] Actually the Durham test. See footnote 8, p. 64.

could be applied also to microscopic sections of the root. Sections of the roots were treated with 50-percent nitric acid and then flooded with 10-percent ammonia. If rotenone was present, an evanescent blue-green color developed as soon as the ammonia had penetrated the cells. The color lasted for only a few minutes; hence close, continuous observation under the microscope was necessary after the addition of ammonia. By using this test, Van der Laan concluded that the active principle (rotenone) in derris root was localized in the parenchyma cells of the pith and medullary rays, in the parenchyma of the wood, and in the parenchyma of the bark. No rotenone was found in the vessels of the wood or in the cork layers of the bark.

Though Buckley (*1*) found that there was apparently no toxicarol in varieties of *Derris elliptica*, and thus made the findings of Van der Laan more specific, Harper (*16*) found that malaccol, another rotenoid of *D. elliptica*, also reacted positively to the Durham test, with an intense blue color changing to green. He postulated that this fact alone showed rings A, B, and C of malaccol to be the same as in rotenone itself. Unless the rotenoids existed as a whole resin in these various portions of the plant, the nonspecificity of Durham's test for rotenone would render Van der Laan's findings even less concrete. Results of the present investigation indicate, however, that rotenone and its congeners are elaborated concomitantly as discrete globules of whole derris resin in certain specific cells found in the xylem rays, in the xylem and phloem parenchyma, and in the pericycle. The cells containing these globules of resin are most dense in two or more concentric rings in the xylem parenchyma. The locations of the rotenone-containing regions are shown in figure 2.

By steam distillation of an extract of the shade-dried leaves of *Tephrosia vogelii*, Hanriot (*15*) isolated an oily liquid which had the reducing properties of an aldehyde. Cahn, Phipers, and Boam (*2 pp. 204, 208, 209*) discussed methods of extracting "fats or rotenone" from *Derris* sp. Goodhue and Haller (*10*) found the noncrystalline part of the extractives from *T. virginiana* to contain a considerable quantity of an oil composed mostly of sesquiterpenes. In the present investigation, an oil which appears to contain at least one terpenelike compound with the reducing properties of an aldehyde was obtained from *D. elliptica*.

The globules of resin found in certain specific cells in *Derris elliptica* are probably in partial solution in a mixture of terpenelike compounds. Thus, since derris resin seems to be an oleo resin, hypothesis (a) (p. 62) is substantiated. The presence of saponins was shown to favor hypothesis (*b*). We may therefore conclude that the resin exists within these specific cells in partial solution as an oleo resin, and that, upon destruction of the cell wall, the saponin influence tends to solubilize, or suspend, these globules of resin in any dispersing phase present.

In 1937, Spoon et al. (*34*) reported that discrete granules of starch could be discerned microscopically in samples of finely ground *Derris* spp.; they reported, furthermore, that the external appearance of these starch granules differed among species and might possibly be used as a criterion for identification. In the same year, measurements of the cells of the woody structures of *Derris* spp. and of *Lonchocarpus* spp. were made and reported by Diakonoff (*4*). In 1939, Guil-

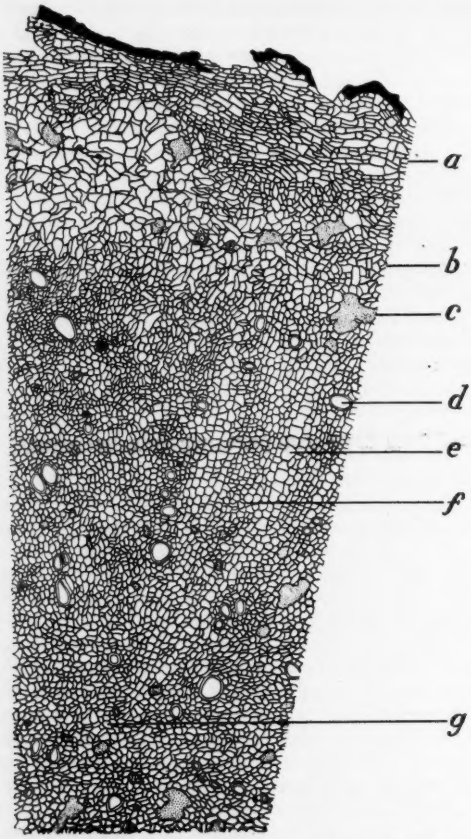

FIGURE 2.—Camera lucida drawing of a transverse section of a root of *Derris elliptica* 4 mm. in diameter. The outer periderm (*a*) and cork contained no rotenone; pericyclic parenchyma (*b*) contained rotenone (secondary phloem not distinguishable); pericyclic fibers, tannin cells (*c*), and secondary xylem vessels (*d*) (small vessels surrounding large vessels, not visible) contained no rotenone; medullary (xylem) ray (*e*) and xylem parenchyma (*f*) contained rotenone, but none was contained in the secondary xylem fibers; fundamental parenchyma (*g*) (center of section) contained rotenone. × 162.

laume and Hervé (*12*) reported that different parts of the same derris root do not contain the same quantity of rotenone, and that small roots are richer in rotenone than large roots.

In a more detailed study, Worsley and Nutman (*41*) reported that resin-containing cells could be distinguished in microscopic sections of derris root by means of the Durham test. They concluded that rotenone occurs first in small cell groups in the secondary cortex opposite the protoxylem when the plant is about 6 weeks old, and that it spreads gradually throughout the xylem parenchyma and cortex. They further stated that starch and rotenone do not occur

in the same cell, but rather in mutually exclusive groups, and that the cells which contain rotenone are apparently unspecialized morphologically. It will be seen that these findings are in accord with those of Van der Laan (*37*), previously mentioned.

Worsley and Nutman (*41*) and Van der Laan (*37*) were probably justified in basing their conclusions almost entirely upon their observations with the Durham color reaction, for Jones and Smith (*22*) had shown previously that this reaction was well established as a rapid qualitative test for rotenone and related compounds. The writers felt, however, that, since the reactions involved were obviously so violent chemically, any microscopic observations following a treatment with strong nitric acid and hydroxide ion (*19*, *29*) could not justifiably be considered as final proof.

In a more recent investigation, Worsley (*39*, *40*) found rotenone in almost every part of *Tephrosia vogelii*, including all the floral organs. In some organs it was present in normal cells, while in others it was present in morphologically distinct cells, often of considerable size. It was found in the seeds of *T. vogelii*, *T. candida*, *T. ehrenbergiana*, *T. toxicaria*, *Derris elliptica*, *D. malaccensis*, *D. uliginosa*, and *Mundulea sericea* (*suberosa*). On germination of the seeds, rotenone either disappeared entirely or diminished greatly in amount, which "suggests that it is of some definite use to the plant." In all parts except the seed, but including the seed testa, rotenone remained undiminished after the plant or organ had withered or died. When kept in the dark until dead, small plants of *D. elliptica* showed a decrease in the rotenone content of their roots. Worsley (*39*), however, was unable to extract a rotenone-decomposing enzyme from the seeds of *Mundalea*. Considerable work on this problem of the role played by rotenone in the plant has been done at the Puerto Rico station by Moore and Jones (*26*).

Only one qualitative reference relative to the tannin content of *Derris* spp. has been found, namely, that of Power (*28*), who found water-soluble gallotannin in *D. uliginosa*. The present work has shown, however, that there are large quantities of tannins in *D. elliptica*, but that in the fresh root these tannins are not appreciably water-soluble. Aqueous ferric chloride solution applied to sections of roots showed the presence of very numerous darkly stained tannin bodies in the outer layer of the pericycle. The colorimetric tests available did not permit of a determination of whether rotenone occurs in these tannin cells also.

Goodhue and Haller (*9*) investigated some of the constituents of aqueous derris extracts qualitatively and reported that the filtered milky suspension so obtained responded to the Goodhue (*8*) modification of the Gross and Smith (*11*) colorimetric test for rotenone. Goodhue and Haller (*9*), furthermore, refer to the aqueous extract of derris root as the "milky suspension of resin." This reference appears to be the only recorded suggestion (cf. Wray (*42*)) that actual globules of whole derris resin are suspended in such an extract, and the authors cite no proof. Using dried root of *Derris* and *Lonchocarpus*, Diakonoff and Smulders (*5*) had demonstrated earlier that there was a direct correspondence between the content of dibutyl ether-extractable substances in these roots and the amount of material which could be extracted by water in the form of a white powder.

The present investigation has shown that the aqueous extract of dried derris roots and both the aqueous extract and the "sap" of fresh derris roots do indeed contain particles (globules) of whole derris resin in colloidal suspension, as shown by their isolation and indentification. These particles (globules) exhibit Brownian movement, range in size from 0.8μ to 3.9μ in diameter, and may be removed quantitatively from the suspension by filtering it through a Seitz filter (pad No. 6). Such suspensions also contain discrete particles (granules) of starch, as shown by standard selective color tests on isolated material. The starch grains range in size from 7μ to 38μ in diameter and may exhibit secondary Brownian movement from impact of the resin bodies. The aqueous "milk" from fresh root contains more starch than that from the dried root.

Goodhue and Haller (*9*), after investigating some of the water-soluble constituents of an aqueous extract of ground dried derris root, reported the presence of both glucose and levulose and saponinlike glucosides which induced foaming but which were nonhemolytic. Worsley (*38*) reported that he made unsuccessful attempts to isolate a saponin from *Mundulea*.

The writers have reinvestigated the sugar content of aqueous extracts of derris roots. Unconcentrated aqueous extracts reduced Fehling's solution after 20 to 30 minutes; acid hydrolysis of these extracts, followed by neutralization, reduced this reaction time to 2 minutes. Thus no uncombined, simple reducing sugars were present, but a disaccharide or a molecule with a sugar moiety was present, and the reducing sugars were liberated by the acid hydrolysis. These sugars were further characterized by the preparation of their mixed osazones; microscopic comparisons with pure specimens indicated the possible presence of fructose (levulose) and the presence of glucose from saccharose (sucrose), and probably xylose in the combined state. The indication from these tests was that the rotenoids do not exist in the plant sap as glycosides.

The unusual physical properties of aqueous extracts of finely ground derris root suggest the presence of large quantities of saponins. Aqueous extracts, when freed of all suspended material, exhibited the following characteristic tests for saponins: Foaming, emulsive, and reducing powers; precipitation with lead acetate; and specific color reactions. When converted into an isotonic salt solution, aqueous derris extract hemolyzed beef erythrocytes upon standing. Accordingly, there are slowly hemolyzing saponins present in derris root. While these substances may exert a synergistic effect upon the efficacy of rotenone as a toxicant, their effect on the state of rotenone in the plant cell could not be determined, although they may conceivably act as solubilizers or mutual solvents.

SUMMARY

Rotenone exists in situ in the form of whole derris resin (extractives) which occurs as discrete particles (globules) in certain cells in the xylem rays, xylem parenchyma, phloem parenchyma, and pericycle, and it does not occur in the vessels or in the fibers. Cells containing rotenone, in the resin form, are most numerous in the parenchyma of the phloem and of the xylem rays, being most dense in two concentric rings in the xylem.

Starch is also present in great quantities in rings in the xylem and phloem parenchyma, apparently alternating with rings of rotenone-containing parenchyma. No starch occurs in fibers or in vessels, and starch and whole derris resin are not contained in the same cells.

These particles (globules) of whole derris resin are in partial solution in an ethereal oil in situ. When the cell wall is broken the globules of resin and oil are suspended, probably by means of slowly hemolyzing saponins which were shown to be present, in the sap of the plant.

The milky sap exuding from freshly cut roots of *Derris elliptica*, and the milky aqueous extracts of either living or air-dried roots, are acidic suspensions (pH 5.5 to 6.0) containing these particles (globules) of whole resin, ranging from 0.8μ to 3.9μ in diameter, and particles (granules) of starch, ranging from 7μ to 38μ in diameter. The former particles exhibit Brownian movement, but both types of particles may be removed quantitatively from the aqueous suspension by passing it through a filter of low porosity.

Although aqueous extracts of derris root contain saccharose, which readily hydrolyzes to fructose and glucose, experimental evidence indicated that the rotenoids do not occur, in situ, in glycosidic combination.

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TOLERANCE OF LAMBS FOR BLACKSTRAP MOLASSES[1]

By H. M. BRIGGS, *associate animal husbandman*, and V. G. HELLER, *head, Department of Agricultural Chemistry Research, Oklahoma Agricultural Experiment Station*

INTRODUCTION

In normal years blackstrap molasses is a common constituent of commercial mixed sheep concentrates as well as of mixed feeds for other classes of livestock. When experiments showed that high levels of blackstrap molasses lowered the digestibility of some nutrients in the lamb ration (*1*, *2*),[2] it was felt that further work should be done to find the critical point at which such reductions occur. Accordingly, two digestion trials were made to determine the tolerance of lambs for blackstrap molasses before a reduction occurred in the apparent digestion coefficients of the respective nutrients.

Previous work at the Oklahoma Station (*1*, *2*) had shown that blackstrap molasses added to the ration of lambs at from 22.5- to 25.2-percent levels, depresses the digestibility of the fat in the ration. The apparent digestion coefficients of protein and nitrogen-free extract were also appreciably reduced, but the coefficient for the apparent digestibility of fiber was not significantly altered. In one trial (*2*) an addition of 10 percent of blackstrap molasses did not materially change the efficiency with which the lambs could digest the various nutrients in the ration.

Mitchell, Hamilton, and Haines (*6*) reported that additions of glucose reduce the ability of calves and lambs to digest protein and fiber and to utilize the energy of the ration. Hamilton (*3*) confirmed these results in studies with lambs. Johnson, Hamilton, Mitchell, and Robinson (*5*) showed that corn molasses lowers the digestion of protein and fiber in the ration of the lamb. Briggs and Heller (*2*) found that sucrose, corn sirup, as well as potassium salts, compounded to resemble the salts of commercial blackstrap molasses, produced noticeable changes in the digestibility of nutrients. An addition of the salt mixture depressed the digestion of all nutrients, but only the apparent digestion of crude fiber was lowered a significant amount. Sucrose materially reduced the apparent digestion of all nutrients except fiber, while corn sirup lowered the apparent digestion coefficients of all nutrients except nitrogen-free extract.

Optimum results with poultry have been secured in rations containing not more than 10 percent of blackstrap molasses. Upp (*10*) found rations containing 10 percent satisfactory for laying hens, but 15-percent levels were decidedly laxative. Ott, Boucher, and Knandel (*7*) presented evidence showing that a 4-percent level of molasses had a slightly stimulating effect on the early growth of chicks. Four- and six-percent levels increased feed consumption, but feed efficiency in producing gain was highest on a basal ration that contained no

[1] Received for publication November 18, 1943.

[2] Italic numbers in parentheses refer to Literature Cited, p. 87.

molasses. These same workers (*8*) found that molasses could be used satisfactorily to the extent of 6 percent to replace yellow corn in the ration of laying hens.

PROCEDURE

Commercial blackstrap molasses was substituted for part of the corn in a basal ration consisting of corn, cottonseed meal, and alfalfa hay. Molasses was used at 5-, 10-, and 15-percent levels with 46, 90, and 136 gm. replacing an equal weight of corn in a basal ration containing 410 gm. of shelled yellow corn. The composition of the rations is given in table 1.

TABLE 1.—*Quantitative composition of the daily rations used in 2 digestion trials with lambs*

Feed	Grams of different feeds used in ration—			
	A	B	C	D
Alfalfa hay	454	454	454	454
Cottonseed meal	44	44	44	44
Corn	410	364	320	274
Blackstrap molasses	------	46	90	136

The two trials were conducted on the same lambs and differed only in that the lambs were approximately 105 days older in the second study than in the first and the trials were carried out under different climatic conditions. The first trial was startted in midwinter; the second was conducted in late spring and early summer. The lambs used were the eight heaviest in a shipment of grade Rambouillet feeders from southwestern Texas. Their average weight was about 80 pounds at the beginning of the first trial and they gained approximately 0.1 pound daily during each trial.

The lambs spent a 10-day preliminary feeding period in a 5- by 4 foot pen and were then placed in a false-bottom metabolism cage for a 10-day collection period. The lambs were fed in open buckets in the pens and in a specially constructed feeder while in the cages; very little feed was lost in either feeder. One-half of the daily feed allowance was given in the morning and the remainder at night. The feeds were weighed on a scale sensitive to 0.1 gm.

The alfalfa hay was ground in a hammer mill and forced through a ¾-inch-mesh screen. The shelled corn was fed whole and the cottonseed meal was of the popular pebble-cake size. The blackstrap molasses was weighed, diluted with water, and added to the other feeds. The sugar determinations on the molasses showed the product to have 44.1 percent total sugar. The sucrose content was 29.0 percent, and 15.1 percent was invert sugar.

Feces were collected once each day and the collection for each lamb was dried at 90° C. for 24 hours. The collection was then weighed and sealed; at the end of the 10-day collection period the dried feces for each lamb were placed in a container, thoroughly mixed, and sampled for analysis.

TABLE 2.—*Percentage composition of feeds used in digestion trials with lambs*

Feed	Water	Protein [1]	Fat	Crude fiber	Ash	Nitrogen free extract
Alfalfa hay	10.97	17.70	1.69	29.98	7.49	32.17
Corn	11.88	8.89	3.19	1.95	1.46	72.63
Cottonseed meal	7.94	42.03	5.69	10.19	5.91	28.24
Blackstrap molasses	34.65	4.98			8.05	52.32

[1] N×6.25.

The chemical composition of the feeds used are given in table 2. The alfalfa hay was U. S. No. 2 grade; the corn was U. S. Sample grade. The same feeds were used in both trials.

RESULTS

Each of the eight lambs used was fed the four rations A, B, C, and D during both trial 1 and trial 2. The apparent digestion coefficients for the rations are given in table 3.

TABLE 3.—*Apparent digestibility (percent) of lamb rations* [1] *containing various levels of blackstrap molasses*

Lamb No.	Protein digestion coefficient for ration—				Fat digestion coefficient for ration—				Crude fiber digestion coefficient for ration—				Nitrogen-free extract digestion coefficient for ration—			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Trial 1:	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
409	74.8	76.8	74.7	70.1	75.4	74.0	65.2	66.7	56.2	57.5	57.5	54.6	87.2	88.0	87.7	85.9
393	73.3	69.3	72.8	72.9	70.0	62.0	66.5	57.3	53.3	50.4	61.4	62.8	85.9	83.9	87.4	87.3
330	74.9	73.2	73.5	73.8	73.8	73.1	70.2	63.1	54.4	56.9	57.5	58.2	86.0	86.8	86.6	85.8
374	77.0	75.3	74.7	74.1	71.5	72.7	56.4	61.3	61.8	56.6	60.7	60.5	89.1	87.9	87.3	87.3
366	73.2	74.6	72.9	74.1	69.4	70.6	75.0	62.6	56.5	57.3	54.5	53.1	87.0	88.2	85.3	85.6
308	75.4	74.3	74.4	74.4	62.5	65.6	68.6	65.9	60.1	60.6	57.2	55.5	87.6	86.8	87.0	86.1
324	74.7	76.2	75.8	70.7	71.5	69.5	72.5	68.9	55.2	53.3	54.0	54.3	88.0	87.1	85.6	83.4
345	72.7	71.4	73.3	71.9	63.2	64.0	65.8	68.7	51.3	59.2	54.8	56.1	86.8	87.6	87.9	86.5
Average	74.5	73.9	74.0	72.7	69.7	68.9	67.5	64.3	57.1	56.5	57.2	56.9	87.2	87.0	86.8	86.0
Trial 2:																
409	70.4	72.5	69.5	76.6	69.8	58.5	61.3	46.3	51.7	57.4	46.7	54.2	86.7	86.4	79.8	82.3
393	71.4	70.7	73.4	71.6	60.7	68.1	61.8	52.9	50.0	46.8	57.1	53.1	83.6	80.3	80.7	83.6
330	74.8	70.4	73.3	66.2	65.7	72.2	57.1	55.6	58.2	46.4	51.7	44.2	84.6	85.4	82.9	78.3
374	74.7	73.4	77.2	75.1	72.6	58.0	65.5	63.6	52.5	56.3	56.6	57.9	84.4	85.3	86.1	83.8
366	74.6	77.0	70.3	74.7	63.0	59.9	67.0	64.0	48.3	58.5	48.7	59.2	82.9	85.0	84.9	85.7
308	72.9	75.6	72.4	70.8	54.4	59.8	47.3	67.5	53.9	53.2	55.5	52.1	83.5	85.1	83.6	81.4
324	75.6	69.5	72.8	67.8	61.5	56.5	62.2	64.6	51.2	40.7	58.7	50.1	84.9	80.8	85.3	84.8
345	75.5	74.2	71.4	68.1	66.1	62.2	70.1	46.8	50.8	58.8	51.4	50.7	86.8	84.0	83.3	83.6
Average	73.7	72.9	72.5	71.4	64.2	61.9	61.5	57.7	52.1	52.3	53.3	52.9	84.7	84.0	83.3	82.9
Average, 2 trials	74.1	73.4	73.3	72.1	66.9	65.4	64.5	61.0	54.6	54.4	55.2	54.9	85.9	85.5	85.1	84.5

[1] A, basal; B, containing 5 percent molasses; C, 10 percent molasses; D, 15 percent molasses.

Table 4 shows the differences between the means of the 16 apparent digestion coefficients secured for each of the 4 rations. Snedecor's (*9*) test for significance was applied to the data of table 3 and the results are summarized in table 4. A value of $P=0.05$ was considered as the significant level; a value of $P=0.01$ was considered highly significant. The average coefficient secured for each ration in both the first and second periods are given in table 3, but they are not treated separately in the statistical analysis.

TABLE 4.—*Summary of the differences between means of apparent digestion coefficients of lamb rations and the significance of the differences* [1]

Rations compared [2]	Protein	Fat	Fiber [3]	Nitr gen-free extract
A and B	0.7	1.5	0.2	0.4
A and C	.8	*2.4	−.6	.8
A and D	*2.0	**5.9	−.3	1.4
B and C	.1	.9	−.8	.4
B and D	1.3	**4.4	−.5	1.0
C and D	1.2	**3.5	.3	.6

[1] *Significant at 0.05 point; **highly significant at 0.01 point; − not significant.
[2] A, Basal; B, containing 5 percent molasses; C, 10 percent molasses; D, 15 percent molasses.
[3] Minus sign indicates that digestion coefficient was less for check ration than for the one with which it was compared.

The feces of the lambs receiving ration D (15-percent molasses) were usually somewhat less dry and less well formed than those of lambs on the other three rations, but they were far from being as soft as those reported for lambs receiving a 25-percent level of molasses (*2*).

DISCUSSION

INFLUENCE OF 5-, 10-, AND 15-PERCENT LEVELS OF BLACKSTRAP MOLASSES ON PROTEIN DIGESTION

In this study each succeeding increase in the amount of blackstrap molasses further lowered the digestibility of the protein in the ration when molasses was substituted for corn in the basal ration of corn, cottonseed meal, and alfalfa hay. While each level of molasses tended to lower the efficiency of protein digestion, the 15-percent level was the only one at which the decrease was significant. The difference between the mean of the 16 coefficients was 2.0 percent whereas a difference of 2.1 percent would have been needed for the difference to be highly significant. The standard error of the difference between these means was 0.71. The lambs were less efficient in protein digestion in the second trial than they were in the first, and all lambs did not react alike to the different rations. This condition was handled statistically not only by analyzing the data for the difference between rations and within rations, but also by removing variations that occurred between the lambs from that within the lambs. In a single preliminary trial (*2*) on seven lambs a 10-percent level of molasses decreased digestibility of protein by 2.9 percent, but the difference was not significant.

It had previously been reported (*2*) that a 25-percent level of molasses lowered by 6.5 percent the digestibility of a ration containing similar basal feeds with almost identical digestibility. This difference was highly significant. It appears from these data on digestibility

that lambs tolerate a 5-percent level of molasses and digest protein with practically the same efficiency at the 10-percent level; higher levels hamper protein digestibility.

THE INFLUENCE OF 5-, 10-, AND 15-PERCENT LEVELS OF BLACKSTRAP MOLASSES ON FAT DIGESTION

In two previous studies (*1*, *2*) it was found that a level of blackstrap molasses approximating 25 percent of the ration caused a very marked reduction in the digestibility of the ether-soluble fraction. Sucrose and corn syrup likewise lowered the apparent digestion coefficients of fat. Neither potassium salts compounded to resemble those found in blackstrap molasses nor a 10-percent level of molasses produced a significant reduction, although in each case a depression was noted. Irwin, Weber, and Steenbock (*4*) have observed that large concentrations of either potassium chloride or calcium chloride decrease the digestion of fat in the rat.

There was a general decrease in the digestibility of the fat component of the ration at each level of blackstrap molasses and the decrease became more marked as the amount of molasses was increased. Molasses at the 5-percent level decreased fat digestion by 1.5 percent, but the difference was not great enough, considering the individual differences of the lambs, to be significant; at a 10-percent level the reduction was 2.4 percent and significant. A highly significant reduction resulted when molasses was used at a 15-percent level, the fat digestion of the ration being 5.9 percent less than that of the basal. The standard error of the difference was 1.06.

It is apparent that molasses cannot be used in excess of 5 percent without decreasing the ability of the lamb to digest the fat in the ration. The reduction observed at the 15-percent level, however, was less than that previously observed at higher levels (*1*, *2*).

INFLUENCE OF 5-, 10-, AND 15-PERCENT LEVELS OF BLACKSTRAP MOLASSES ON CRUDE FIBER DIGESTION

In this study, blackstrap molasses did not noticeably affect apparent crude fiber digestion coefficients at the three levels at which it was fed. There was a slight tendency for the coefficients to be higher at the 10-percent level than at the lower levels or at the 15-percent level. Fiber digestion was improved 0.6 percent when 10 percent of molasses replaced a like amount of corn in the basal ration. However, the standard error of the difference was 1.62 and the difference was not significant. In similar studies at this station (*1*, *2*) very slight reductions in fiber digestibility have followed the use of larger amounts of blackstrap molasses than were used in these studies, but the differences were not significant.

INFLUENCE OF 5-, 10-, AND 15-PERCENT LEVELS OF BLACKSTRAP MOLASSES ON NITROGEN-FREE-EXTRACT DIGESTION

The trend of the results obtained confirm observations of previous studies (*1*, *2*) that lambs do not make as ready use of that part of the ration classed as nitrogen-free extract when blackstrap molasses is included in the ration as they do a basal ration that contains no molasses. However, at the low levels of molasses used, the reductions were not significant. When molasses was fed at the 15-percent

level, it decreased the digestibility of nitrogen-free extract 1.4 percent; the standard error of this difference was 0.8 and the difference was not significant. Previous studies (*1*, *2*) have shown that 25-percent levels of blackstrap molasses did lower nitrogen-free-extract digestion considerably while a 10-percent level did not.

SUMMARY

Eight wether lambs were used in two digestion trials to determine at what level of blackstrap-molasses feeding the apparent digestibility of the ration is altered. The molasses was substituted for corn at 5–, 10–, and 15-percent levels in a basal ration of corn, cottonseed meal, and alfalfa hay.

The ability of the lambs to digest protein was not influenced measurably except at the 15-percent level. Apparent fat digestion coefficients were reduced at both the 10- and 15-percent levels. Neither apparent fiber digestion coefficients nor those of nitrogen-free extract were influenced significantly by molasses at any of the levels used.

It appears from this study that blackstrap molasses can be used most effectively for lambs, so far as digestibility of the ration is concerned, when it constitutes not more than 5 percent of the ration. However, digestibility was not so greatly reduced at the 15-percent level but that it would often be economical to use the product at such a level in commercial lamb feeding.

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